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Contrasting Effects of Polycations on Plaquing Efficiency of Encephalomyocarditis Virus Variants

J. E. CRAIGHEAD AND C. H. LAYNE

Department of Pathology, College of Medicine, University of Vermont, Burlington, Vermont 05401

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Polycation treatment of L cell monolayers affected plaquing efficiency of both the r^+ and r variants of the encephalomyocarditis virus. Plaque formation by r^+ variant was decreased markedly by three structurally different types of synthetic basic polymers, diethylaminoethyl dextran, hexadimethrene (polybrene), and basic polyamino acids. In contrast, these same substances increased substantially the number of plaques formed by the r variant. The effect on the two variants was observed when polycations were applied to the cells before or simultaneously with the introduction of virus. The molar concentration and size of the polymer proved important. Thus, basic polyamino acids of low molecular weight were significantly more inhibitory for the r^+ variant than were those of high molecular weight. On the other hand, plaquing efficiency of the r variant was increased by relatively large polyamino acids, but not by polymers of small size. Basic polyamino acids inhibited r^+ plaque formation to a greater degree at low than at high *p*H values. However, plaquing efficiency of the r variant in polycation-treated cultures was not affected by changes in *p*H. Basic polymers appear to bind to cell membranes and affect either attachment or uptake of the viruses. The evidence suggests that the substances influence by different mechanisms the interaction of the r^+ and r variants with cells.

Synthetic cationic polymers and naturally occurring basic polypeptides of a variety of types enhance uptake of foreign protein and nucleic acid by cultured cells (13, 16, 18, 22, 23). These polycations also affect the interaction of intact virus with cells. Protamine and diethylaminoethyl (DEAE) dextran increase the proportion of cells infected with rabies virus (12). Poly-L-lysine and protamine enhance adsorption of fowl plague virus (1), and DEAE dextran stimulates uptake of respiratory syncytial virus (17) and avian sarcoma virus (26). In contrast, protamine inhibits attachment of mengovirus to L cells (6). Histones and protamine reduce plaque formation by Semliki Forest virus (7), and poly-L-ornithine has a similar effect on Sindbis virus (25). The mechanism by which polybasic substances affect the interrelation between cells and macromolecules or viruses is not clear. Relatively high concentrations reversibly damage cell membranes, whereas low concentrations stimulate pinocytosis (21). Basic polymers and polypeptides appear to interact with negatively charged membranes of culture cells (11, 19). Pagano and Vaheri (18) suggested that DEAE dextran complexes with poliovirus ribonculeic acid and the cell surface and thus effects attachment and subsequent "viropexy." Observations by Allison and Valentine of vaccinia and fowl plague viruses are consistent with the concept of electrostatic binding of virus particles to the cell by polycations (1).

Takemoto and Liebhaber described two plaque variants of encephalomyocarditis (EMC) virus, r^+ and r, which differ in sensitivity to sulfated acid polysaccharide inhibitors (24). The r^+ variant is bound electrostatically by the polyanions and forms only small plaques under agar; the r variant, which yields relatively large plaques, is unaffected by the inhibitors at equivalent concentrations. Although incomplete, the available information suggests that the differences between the two variants are a reflection of the amino acid composition and ionic state of their protein capsids (15).

Recorded here are studies which compare the effects of three types of synthetic polycations on the interaction of the EMC variants with L cells. It has been found that the polybasic substances increase the plaquing efficiency of r, but markedly

reduce plaque formation by r^+. The evidence suggests that basic polymers bind to the cell membrane and differently affect attachment or uptake of the two viruses.

MATERIAL AND METHODS

Cell cultures. Monolayers of L-929 were grown in plastic dishes or glass tubes using Eagle's medium containing 10% heated (56 C, 30 min) calf serum and maintained in Eagle's medium supplemented with 5% heated chicken serum. Penicillin, streptomycin, kanamycin, and nystatin were incorporated in all media.

Virus. The r^+ and r variants of EMC virus were provided by K. K. Takemoto of the National Institutes of Health. Pools were prepared in L cells from selected plaques. Studies with the r^+ variant were done in 35-mm plate cultures with a virus inoculum of 0.2 ml; 60-mm plates with an inoculum of 0.5 ml were employed for the r variant. Except as noted, from 50 to 100 plaque-forming units (PFU) of the two viruses and adsorption periods of 30 or 60 min at room temperature were used. The agar overlay medium and methods of conducting assays have been described (8).

Polycations. Solutions of DEAE dextran [molecular weight (MW) = 2×10^6; Pharmacia, Inc., Uppsala, Sweden] and Polybrene (MW = 3.6×10^5; Abbott Laboratories, North Chicago, Ill.) were prepared in maintenance medium adjusted to *p*H 7.4. Poly-L-ornithine (PLO; MW = 2×10^5, 9×10^4, and 4.5×10^4), poly-L-lysine (PLL; MW = 1.95×10^5, 9.2×10^4, and 3.8×10^4) and poly-D-lysine (PDL; MW = 1.1×10^5 and 1.9×10^4) were obtained from Pilot Chemical Co., Watertown, Mass., and prepared in Hanks' balanced salt solution at *p*H 7.4 or in isotonic 0.045 M PO_4-saline buffer over a *p*H range of 6.8 to 8.0. Cultures were either treated with the polybasic substance before the addition of virus or exposed to virus diluted in a polycation-containing solution. In the pretreatment experiments, 35- and 60-mm plates received 2 and 5 ml, respectively. After incubation with the polycation for the period indicated below, the monolayers were washed and virus was added. When polycation and virus were introduced into the plates simultaneously, the monolayers were washed before the addition of the agar overlay.

RESULTS

Monolayer pretreatment with DEAE dextran and polybrene. Plaquing efficiency of both the r^+ and r variants was affected when monolayers of L cells were exposed to polycations before the addition of virus (Fig. 1). In polycation-treated cultures, the number of r^+ plaques was reduced substantially, whereas the number of r plaques was increased. The effect on both variants was observed when monolayers were treated for periods as brief as 5 min with medium which contained 5 to 50 μg of either DEAE dextran or polybrene per ml. The relative increase or decrease in the number of plaques formed by the two variants did not correlate with the polycation dosage

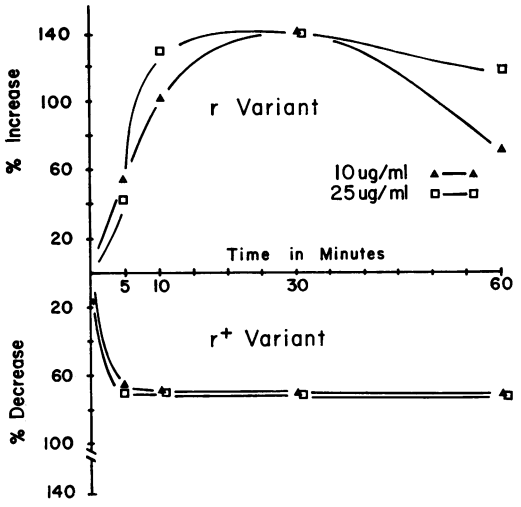

FIG. 1. *Effect of pretreatment of cell monolayers with polybrene on plaquing efficiency of r^+ and r variants. Cultures were incubated with the polycation-containing medium for the indicated period before the introduction of virus. Virus adsorption was allowed for 60 min before application of the agar overlay.*

over this range. Indeed, it usually was maximal at concentrations of 5 μg/ml. The effectiveness of the basic substances was not enhanced by extending the treatment period for longer than 10 to 20 min (Fig. 1).

Plaque counts of the r^+ variant in monolayers that had been pretreated with polycation were not increased by prolonging the time allowed for virus adsorption. Exhaustive washing of the cultures also failed to affect results. Thus, the action of DEAE dextran and polybrene on cell susceptibility was irreversible. The number of detectable r^+ PFU was not reduced when monolayers were treated with the polybasic substances 20 min after the inoculation of virus. As will be shown below, this adsorption period is sufficient to give maximal r^+ PFU in untreated L cells. These findings suggest that polycations do not alter the virus-cell interaction once attachment has occurred.

Monolayer pretreatment with basic polyamino acids. In preliminary studies of the type described above, basic polyamino acids of various molecular weights were found to differ in the effectiveness with which they inhibited plaque formation by the r^+ variant. This observation was investigated systematically using PLL, PDL, and PLO over a range of molecular weights (Fig. 2). At several different concentrations, the polyamino acids of low molecular weight were the most effective. Indeed, polycations of large size exhibited a significant inhibitor action only when relatively high concentrations were employed. Figure 3 depicts the

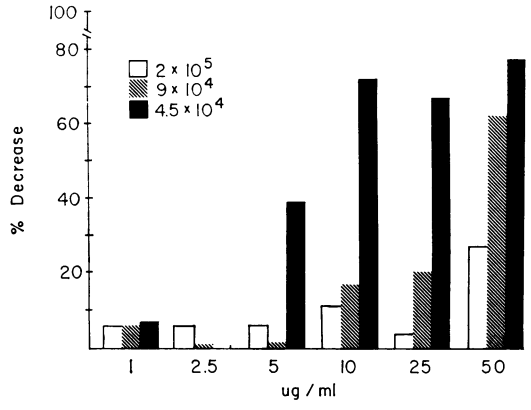

FIG. 2. *Effect of pretreatment of cell monolayers with PLO on plaquing efficiency of r+ variant. Polymers of three different molecular weights were used over a range of concentrations. Cultures were incubated with the polycation-containing medium for 60 min before the introduction of virus. Virus adsorption was allowed for 30 min before application of the agar overlay.*

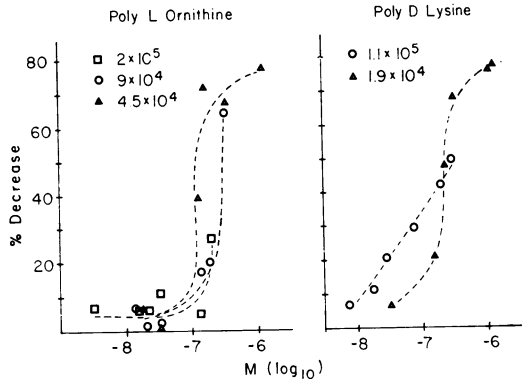

FIG. 3. *Effect of pretreatment of cell monolayers with PLO and PDL on plaquing efficiency of r+ variant, based on molar concentration. Cultures were incubated with the polycation-containing medium for 60 min before the introduction of virus. Virus adsorption was allowed for 30 min before application of the agar overlay.*

inhibition of r+ plaque formation by PLO and PLL, calculated on a molar basis. These data suggest that the number of particles of the polybasic substances used to pretreat the cultures may be a major determinant of effectiveness.

Similar experiments were carried out using the r variant. In contrast to results with r+, only basic polyamino acids with molecular weights of approximately 2×10^5 or greater consistently enhanced plaque formation (Fig. 4). Interestingly enough, the increase in r plaquing efficiency was found at concentrations 10-fold lower than those which inhibited the r+ variant. At concentrations greater than 5 μg/ml, the effect of the polyamino acids was reduced substantially.

Assuming their action is related to the net charge of the molecule, it might be expected that basic polyamino acids would be more effective when NH_3^+ groups are dissociated. To test this possibility, cells were treated with polyamino acid solutions at various *p*H values over a range of concentrations. The monolayers then were washed and inoculated with virus at the same *p*H. Both PLO and PLL inhibited plaque formation of r+ at *p*H 7.4 or lower, but had little or no effect at *p*H 8 or above (Fig. 5). Results were similar when *p*H was adjusted with either phosphate buffer or bicarbonate. Comparable experiments were carried out using the r variant. These studies repeatedly failed to define consistent differences in plaquing efficiency associated with changes in *p*H.

Virus adsorption from polycation solutions. In the work described above, an effort was made to remove residual polycation by thoroughly washing monolayers before the introduction of virus. Additional experiments were undertaken to determine the effect of DEAE dextran and polybrene on the kinetics of virus attachment by inoculating the polycation simultaneously with the virus. Preliminary to these studies, the rate of uptake of the two variants by monolayers was defined (Fig. 6). Repeated experiments showed that the number of r+ plaques reached their maximum 15 to 30 min after inoculation. In contrast, uptake of r variant was slower; maximal plaque counts were obtained only after adsorption periods of 120 to 180 min. These findings are similar to results obtained by Colter et al. (5) with their S and L variants of mengovirus.

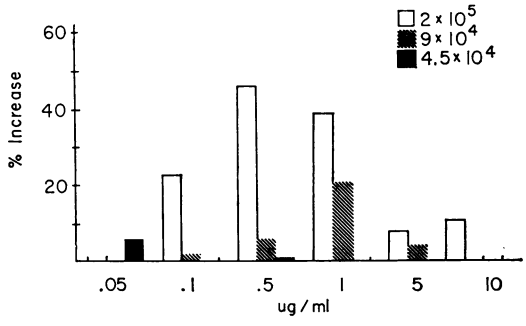

FIG. 4. *Effect of pretreatment of cell monolayers with PLO on plaquing efficiency of r variant. Polymers of three different molecular weights were used over a range of concentrations. Cultures were incubated with the polycation-containing medium for 60 min before the introduction of virus. Virus adsorption was allowed for 60 min before application of the agar overlay.*

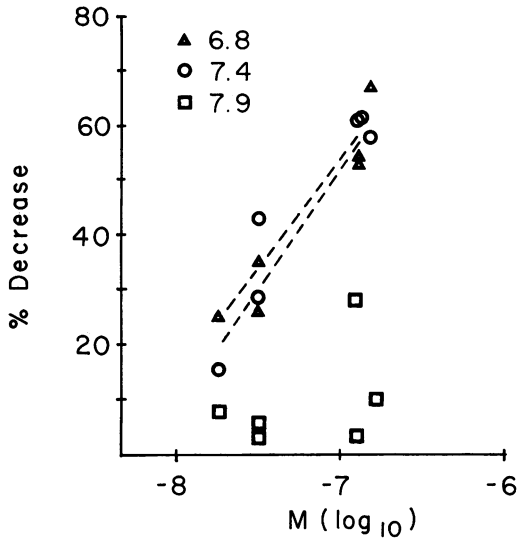

FIG. 5. *Effect of pretreatment of cell monolayers with PLO at various pH on plaquing efficiency of r^+ variant, based on molar concentration. The pH of the polycation solution and virus inoculum was adjusted with phosphate buffer. Cultures were incubated with the polycation-containing medium for 60 min before introduction of virus. Virus adsorption was allowed for 30 min before application of the agar overlay.*

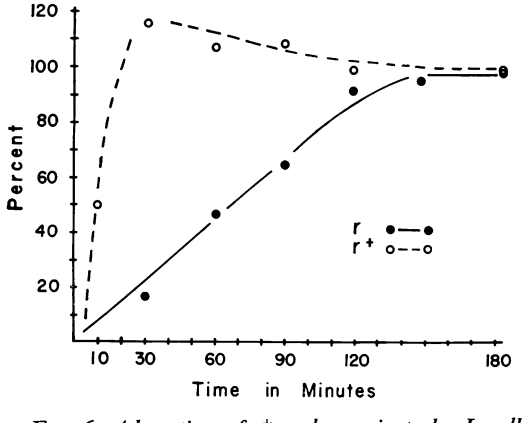

FIG. 6. *Adsorption of r^+ and r variants by L cell monolayers. Cultures were incubated with virus for the indicated period before the application of the agar overlay. Inoculum volume for r^+ = 0.4 ml in 35-mm plates; r = 1 ml in 60-mm plates.*

Plaquing efficiency of the r^+ variant was reduced substantially when virus diluted in a polycation solution was introduced onto monolayers (Fig. 7). This inhibitor effect was observed after adsorption periods of 10 min, and it persisted even when virus was exposed to the monolayer for as long as 180 min. With the r variant, plaquing efficiency was increased when polycations were incorporated in the virus inoculum. However, the effect did not become apparent until an adsorption period of 60 min or more had elapsed (Fig. 8). By 180 min after inoculation, the number of plaques detected in cultures receiving virus in a polycation solution was significantly greater than the PFU developing in control cultures. Thus, under these circumstances, the polycations seemed to act either by increasing the number of effective interactions between virus and cells or by promoting cell uptake of virus.

Polycation effect on virus growth. Figure 9

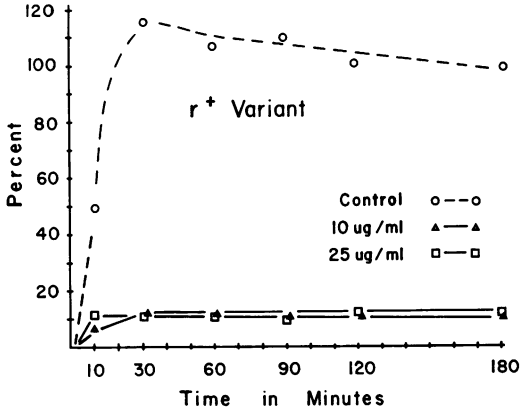

FIG. 7. *Effect of polybrene on plaquing efficiency of r^+ variant. Virus was diluted in the polycation-containing medium and allowed to adsorb for the indicated period before the application of the agar overlay. Inoculum volume = 0.4 ml in 35-mm plates.*

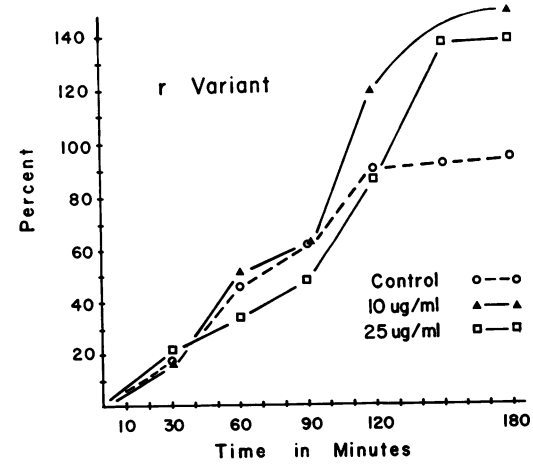

FIG. 8. *Effect of polybrene on plaquing efficiency of r variant. Virus was diluted in the polycation-containing medium and allowed to adsorb for the indicated period before addition of the agar overlay. Inoculum volume = 1 ml in 60-mm plates.*

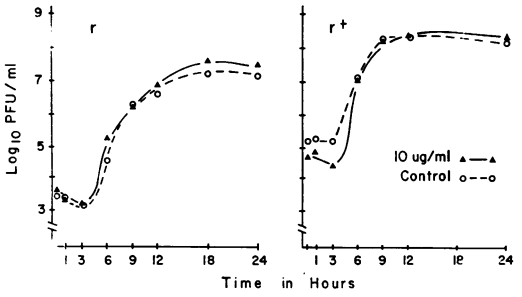

FIG. 9. *Growth of r+ and r variants in the presence of DEAE dextran. Tube cultures were inoculated with 4 PFU/cell of r and 2 PFU/cell of r+ and incubated for 60 min. Cell sheets then were rinsed five times and maintenance medium containing 10 μg of DEAE dextran per ml was introduced. Cultures were held at 36 C and three tubes were frozen at 3-hr intervals. After an additional freeze-thaw cycle, the debris was removed by centrifugation. Controls were treated in an identical fashion except that the polycation was not incorporated in the medium. Before titration, control and test specimens were diluted so that the DEAE dextran concentrations were equal. The diluting fluid, incubated with uninfected cultures, consisted of either DEAE dextran-containing medium for the controls or standard maintenance medium for the test specimens.*

depicts curves for single-cycle growth of the r+ and r variants in L cells bathed by a medium containing DEAE dextran. The polycation was added after the cells had been exposed to high multiplicites of viruses for 60 min. No effect on the total virus yield was observed. Identical results were obtained when polybrene was used.

Fate of unadsorbed virus. An effort was made to determine whether the r+ virus paricles which fail to form plaques in DEAE dextran and PLO-treated monolayers are recoverable from the inoculum after the adsorption period. The number of PFU which were not adsorbed after a 60-min exposure to a pretreated monolayer was assayed by removing the inoculum and transferring it to a fresh culture. The plate was then rinsed thoroughly, and the fluid, also, was tested for virus. In repeated experiments, the total number of PFU recovered from the initial inoculum and the washings was insufficient to account for the plaques not detected in the polycation-treated cultures. Indeed, after the adsorption period, the amount of virus remaining in the inoculum applied to treated and untreated monolayers was roughly comparable. No evidence was found to indicate that the polycation was released by the treated cells into the inoculum or washing fluid.

Polycation cell interaction. Basic macromolecules have been shown to adsorb to erythrocytes by binding electrostatically with the negatively-charged limiting membranes (19). Using packed L cells, efforts were made to remove from a DEAE dextran solution the substance which inhibited plaque formation by the r+ variant, presumably, the polycation. Five extractions, with a total of approximately 7.25×10^8 cells, removed the inhibitor from 5 ml of a solution containing 10 μg of DEAE dextran per ml.

Both DEAE dextran and polybrene altered the integrity of the cell membrane when monolayers were exposed to relatively high concentrations or to lower concentrations for prolonged periods of time (Table 1). Although systematic studies were not carried out, DEAE dextran appeared to be more toxic than the basic polyamino acids. Under the conditions of the experiments described here, toxic effects were not observed in cytologic preparations of the monolayers and in repeated eosin exclusion tests.

DISCUSSION

The r+ variant of EMC virus is bound electrostatically by synthetic and naturally occurring sulfated polyanions. This interaction renders the virus noninfectious and occurs independent of the cell (24). At equivalent concentrations, polyanions fail to bind the r variant. It seems likely that differences in the amino acid composition of the protein capsid of the two variants are responsible for these distinctive properties (15).

Several groups of investigators have isolated and characterized variants of EMC which are similar to the viruses used in this study (20–22). Work by Colter and his associates with the S (small), M (medium), and L (large) plaque variants of mengovirus parallel in part studies reported here. Like r+, the S and M variants are inhibited by sulfated polysaccharides in agar. Colter et al. (6) found that protamine reduced

TABLE 1. *Effect of pretreatment of L cells in monolayers with polycations on eosin*[a]

Polycation	Amt	Hours of pretreatment		
		1	24	48
	μg/ml			
Polybrene	100	0	2+	3+
	50	0	0	0
	25	0	0	0
	10	0	0	0
DEAE dextran	50	1–3+	4+	4+
	25	0	1–2+	4+
	10	0	0	0

[a] Monolayers were graded microscopically on a scale of 1 to 4+. All, or the majority, of the cells contained eosin in 4+ cultures.

attachment of all three variants to L cells. However, the inhibitory effect of this basic polypeptide on the S variant was 10-fold greater than on the M and L. These workers suggested that the three viruses differed one from another with respect to specific sites of attachment on the cell.

In our experiments, polycations inhibited r^+ plaque formation by affecting the interrelation of the virus with the cell. Although differing as to the nature of their repeating units, NH_3^+ group arrangement, tertiary structure, and molecular weight, each of the three types of basic polymers might be expected to interact with negatively charged components of the cell membrane. Accordingly, it seems reasonable to hypothesize that polycations prevent attachment of virus to the cell by masking or competing for binding sites, possibly sialic acid or a neuraminidase-sensitive acid polysaccharide (14). At equivalent concentrations, basic polyamino acids of low molecular weight were more effective inhibitors of r^+ than those of high molecular weight (Fig. 2). The evidence suggests that the concentration, as well as the size of the polycation particles, is important.

An alternate hypothesis that may account for the inhibitory effect of polycations on r^+ seems worthy of consideration. Since the r^+ variant is bound electrostatically by polyanions in solution, it is likely that the virus capsid possesses a net positive charge. Such being the case, one might expect positively charged polymers on the cell surface to repel particles of a similar charge. As observed in our experiment (Fig. 5), the effect would be more prominent at low *p*H when NH_3^+ groups are dissociated. This possibility is not inconsistent with the concept of a cell surface receptor which acts independent of the charge of the virus capsid.

The fate of the r^+ virus particles that fail to form plaques in polycation-treated cells remains to be elucidated. Fenwick and Cooper (10) showed that poliovirus particles are not infectious after elution from the cell, possibly because they bind with fragments of the membrane. A similar event could occur when and if r^+ interacts with the cell surface. Polycations stimulate the appearance of lysosomal acid hydrolases in the cytoplasm (16). Bukrinskaya et al. (4) showed that production of fowl plague virus is reduced when a basic protein, such as a histone, is added to cultures after the cells have been infected. Thus, r^+ particles may be taken up by polycation-treated cells, yet be inactivated or fail to replicate.

The hypotheses cited above obviously fail to account for the action of polycations on plaque formation by the r variant. An alternate mechanism seems likely. Since the basic polymers act directly on the cell to enhance uptake of foreign material, this effect could be responsible for increased plaquing efficiency. Ryser showed that polyamino acids and DEAE dextran of high molecular weight stimulate pinocytosis of albumin more effectively than do those of low molecular weight (20). Our results are consistent with his finding (Fig. 4). Moreover, the range of the effective polycation concentration for the virus and albumin are comparable (22). The possibility exists that polycations influence the electrostatic interaction of r variant particles with the cell. Since r fails to bind with acid polysaccharides, it is reasonable to suggest that its capsid possesses little or no charge or a net negative charge. Accordingly, basic substances could attract virus to the cell. Such an event may account for the observed increase in the rate of adsorption of r to polycation-pretreated monolayers.

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