Enhancement of Vesicular Stomatitis Virus Following Adsorption with Poly-L-ornithine* (33807)

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Polycations have been found to enhance the uptake of macromolecules by cells in tissue cultures. The uptake of bacterial RNA and DNA (1), infectious viral RNA (2), synthetic double stranded RNA (3), protein (4, 5), and inulin (4) have each been enhanced by the presence of suitable concentrations of polycations. Polycations have also been shown to enhance the uptake of some virus particles (6–9) but not others (10, 11). In the course of studies on the effect of polycations on the titers of representative strains of several virus families, vesicular stomatitis virus (VSV) was enhanced several-fold in titer by low concentrations of the polycation, poly-L-ornithine (PLO). The results of a more detailed study of the mechanism of that enhancement are presented in this paper.

Materials and Methods. Tissues. Monolayers of primary chick embryo fibroblasts (CEF), obtained by trypsinization from 10- or 11-day-old chick embryos, were prepared in 60-mm Falcon tissue culture dishes and used after 24–48 hr when the tissue became confluent. Human muscle–skin diploid fibroblasts (MSF) in generations 10 to 30 were used 48–72 hr after seeding in similar dishes when the monolayers had become confluent and were in the stationary phase.

Media. The CEF were grown in Geys–Tris solution with 5% lactalbumin hydrolysate, 5% heat-inactivated calf serum, 150 μg/ml of potassium penicillin G and 250 μg/ml of streptomycin sulfate. The MSF were grown in Leibovitz medium supplemented with 20% fetal calf serum (FCS), 0.03% glutamine, 0.09% arginine, and the same antibiotics. Media used as diluent in the experiments were similar to the respective growth media but lacked serum.

Virus. The Indiana strain of VSV was obtained from A.C. Allison (National Institute for Medical Research, London); a single stock of culture was prepared, distributed into capillary tubes, and stored at −80°.

Virus titration. Virus was diluted to a concentration twice that desired in the final sample. Equal volumes of the diluted virus and either diluent or serial dilutions of PLO were placed in a series of tubes. From each of these mixtures 2 ml was delivered to each of 4 monolayers which had first been washed twice with 2 ml of diluent. The monolayers were exposed to the mixtures for 60 min at 37° to permit adsorption of virus, after which the cells were again washed twice with 2 ml of dilutent. Each monolayer then received 6 ml of overlay containing 0.9% Bacto Nobel special agar and the ingredients of the respective growth media except that Leibovitz overlay medium had 10% FCS and 0.01% $NaHCO_3$ and Geys–Tris solution had 0.04% $NaHCO_3$. The monolayers were incubated at 37° in air for 24 hr and then overlayed with 2.0 ml of similar agar containing 0.025% neutral red. Plaques were counted after additional 24–48 hr incubation at 37°. PLO was used as a crystalline powder, mol wt 140,000 (Pilot Chemical Company, Watertown, Mass.).

Toxicity studies. Suspensions of CEF and MSF were seeded into tissue culture tubes containing threefold concentrations of PLO ranging from 0.36–90 μg/ml (final). They were incubated at 37° on a stationary rack and inspected daily for attachment of the cells, growth and quality of the monolayers.

Results. The results of titrations of VSV performed in chick and human cell cultures in the presence of varying concentrations of

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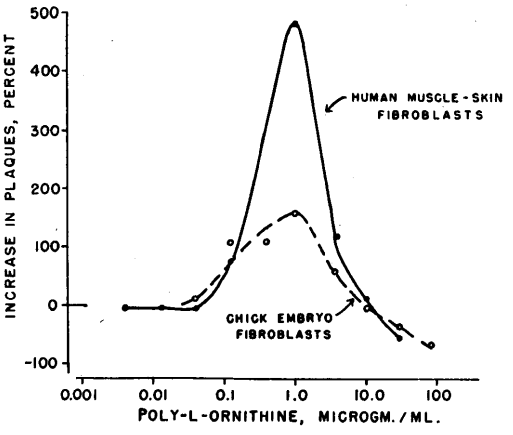

FIG. 1. Poly-L-ornithine enhancement of VSV.

PLO during the adsorption period are shown in Fig. 1. In this figure the percentage of increase in the number of plaques in the culture exposed to PLO over that in the controls adsorbed without PLO is plotted against the log of the concentration of PLO. With both human and chick cells there was a marked enhancement in titer of infective virus, the peak effect being at 1.0 μg/ml. Increasing concentrations beyond 1.0 μg/ml produced decreasing enhancement up to 10 μg/ml, and still higher concentrations resulted in inhibition. When the monolayers of either cell type were grown in the continued presence of PLO, using inocula of cells which provided 90% monolayer formation at 72 hr in controls, there was no difference in the appearance of the tissue in the controls and in cultures made in the presence of threefold concentrations of PLO up to and including 3.3 μg/ml. Similar monolayers resulted with 10 μg/ml but a small amount of debris appeared on the surface, and still higher concentrations of PLO resulted in complete failure of cell proliferation. Thus the enhancement of virus titer of PLO represented in Fig. 1 was not associated with detectable cell toxicity, even in the early part of the declining phase of the curves.

In the following studies of the mechanism of the enhancement of virus titer, MSF were used with PLO in a concentration of 1.0 μg/ml unless otherwise noted and all experiments were done in quadruplicate.

To determine whether the enhancement of infective virus titer of PLO was related to its cationic nature, a polyanion, heparin, was administered simultaneously with PLO during the virus adsorption period. The resulting effect on titer, given as number of plaques, is shown in Table I. As shown, in the absence

TABLE I. Inhibition of PLO Effect on Virus Titer by Heparin.

Conc of PLO (μg/ml)	Conc of heparin (μg/ml)			
	0	1	10	100
	No. of plaques			
0	7	8	4	2
0.1	7	5	8	6
0.3	7	8	5	6
1.1	40	74	8	9
3.3	35	51	19	8

of heparin, enhancement of plaque count occurred with PLO concentrations of 1.1 and 3.3 μg/ml. Heparin had no inhibitory effect on the number of plaques in the presence of 0.3 μg of PLO/ml or less, but in a concentration of 10 μg/ml of heparin, the enhancement of 1.1 μg of PLO/ml was negated and that of 3.3 μg of PLO/ml was reduced. With heparin in a concentration of 100 μg/ml there was no enhancement of VSV titer with any of the concentrations of PLO tested. Heparin at 1.0 μg/ml may possibly have facilitated the enhancement of both 1.1 and 3.3 μg PLO/ml.

Since the charge on PLO is apparently an essential element in the enhancement of VSV titer, an attempt was made to determine whether only enhancement of physical attachment is involved or whether some energy utilizing process involved in cell membrane penetration must take place during exposure to PLO for enhancement to occur. Monolayers were exposed to the virus with and without PLO at 4 and 37° for 60 min. Then the virus preparations were removed, the monolayers were washed two times with diluent, and agar containing overlay was applied. For the monolayers that were treated with virus at 4°, the first wash was performed at 4° with diluent at the same temperature. All the other washes were carried out at room temperature with diluent at 37°.

The results are given in Table II. In the

TABLE II. Effect of PLO on Adsorption of VSV at 4 and 37°.

PLO (μg/ml)	No. of plaques 4°	37°
1.0	59	51
0	10	27

absence of PLO, the lower plaque numbers at 4°, as compared with those at 37°, were expected from the decreased diffusion associated with increased viscosity of the medium at the lower temperature (12, 13). At both temperatures there was enhancement of plaques to a comparable absolute number. This result suggests that it is the physical forces of attachment rather than the energy requiring steps of penetration that are enhanced.

The rate of virus adsorption to a monolayer in the presence or absence of PLO was then studied over a period of 60 min. Monolayers were exposed to virus preparations for varying precise intervals following which the preparations were immediately removed and the monolayers were washed two times with diluent. Agar containing overlay was then applied in the usual manner. The number of plaques plotted vs time are shown in the left panel of Fig. 2. the initial points on the PLO and control curves, recorded at 5 sec, already show a marked difference. The straight-line fit for the control points indicates a zero order reaction. The PLO curve as plotted has two slopes, each of which is greater than that of the control. Thus, after 5 min of very rapid attachment, the rate of attachment in the presence of PLO is constant and remains greater than that of the control. The right panel in Fig. 2 shows the number of plaques plotted vs the square root of time. The control points here are fit by a curve with concavity upward while the values with PLO are fit by a straight line. The kinetic data indicate that adsorption in the presence of PLO behaves as a negative first order reaction.

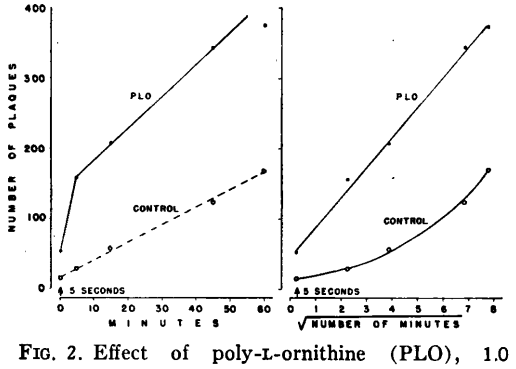

FIG. 2. Effect of poly-L-ornithine (PLO), 1.0 μg/ml, on adsorption of VSV.

To determine if PLO enhancement is affected by the amount of the virus exposed, 3 concentrations of virus were used with and without PLO present during the adsorption period; the results are given in Table III. For each inoculum of virus there was a greater plaque count with PLO than without, and the ratio of plaque counts in the treated to control groups was remably constant. Thus, over the range of virus concentrations studied, PLO enhancement was not a function of virus concentration.

To study the effect of PLO applied to the virus prior to the virus adsorption period, 100-fold concentrated virus suspensions with or without PLO were incubated in a water bath at 37° for 1 hr. Both samples were then diluted 1:100 to achieve countable numbers of plaques and to reduce the concentration of PLO below enhancing levels and the virus suspensions were then exposed to monolayers for 1 hr. The results, shown in Table IV, indicate that pretreatment of virus with PLO did not increase the number of plaques although a control in which PLO was present during the adsorption period gave the expected enhancement.

The effect of pretreatment of cells with PLO prior to adsorption of virus was then

TABLE III. Influence of Size of Inoculum of Virus on PLO Effect.

No. of plaques[a]		
PLO (1.0 μg/ml) (A)	No PLO (B)	Ratio: A/B
292.7	64.7	4.5
88.3	19.7	4.5
16.0	3.7	4.3

[a] Mean of 4 tests.

TABLE IV. Effect of Time of Exposure of Virus to PLO on Adsorption to Cells.

Treatment	No. of plaques	Ratio
A. Virus exposed to PLO[a] before adsorption[b]	261	
B. Control, no PLO	245	A/B = 1.07
C. Virus exposed to PLO[c] during adsorption	436	
D. Control, no PLO	247	C/D = 1.77

[a] One μg/ml for 1 hr applied to concentrated (100×) suspension of virus.
[b] Samples diluted 1:100 before adsorption.
[c] One μg/ml for 1 hr; virus not concentrated.

studied. Cell monolayers were treated with and without 1.0 μg of PLO/ml for 1 hr, then washed twice and challenged with virus. As shown in Table V, there was definite enhancement of plaques in the treated cultures but to a lesser extent than in the control cultures in which PLO was present during virus adsorption. Thus, pretreatment of the cells alone resulted in an appreciable enhancement of virus titer, but less than when adsorption took place in the presence of PLO.

Finally, the effect of applying PLO after the virus adsorption period was investigated. Cells which had been exposed to virus for 1 hr were washed twice with diluent and then treated with and without PLO for 1 hr. The PLO-treated cells yielded a slightly larger number of plaques as compared to the untreated cells (Table V). Thus, treatment of cells after adsorption of virus with PLO gave minimal, if any, enhancement.

Discussion. The accumulating data show that polycations affect the uptake of macromolecules and virus particles by cells in a variety of ways. To understand the differences, it is necessary to consider both the range of moieties tested and the types of detection systems used to measure uptake. Bacterial RNA and DNA as well as infective viral RNA and synthetic double stranded RNA are long polymers with sugar phosphate backbones having multiple negative charges at neutral pH (14). The proteins which have been studied, albumin (4) and interferon (5), are polypeptides with an isoelectric point below pH 7.0, resulting in a small net negative charge at physiological pH. Viruses are much larger structures with isoelectric points around pH 5.5 (13). Presumably they all have a net negative charge at physiological pH. Some have a presumably homogenous outermost layer of protein whereas the protein coat of others is encapsulated in a membrane containing large quantities of lipid as well as protein (15). It is known that at least some of the viruses have specific combining sites in their outermost layer which attach to receptor sites on susceptible cells.

The systems used to detect uptake can be divided into two classes: physical and biological. The physical systems utilize monitoring of radioactivity of labeled RNA (1) or albumin (4) taken up by cells. The biological procedures involve bioassay of infectivity following uptake of infectious RNA (2) or of

TABLE V. Effect of Time of Treatment of Cells with PLO on Growth of VSV.

Treatment	No. of plaques	Ratio
A. Cells exposed to PLO[a] before adsorption of virus[b]	49	
B. Control, no PLO[b]	24	A/B = 2.04
C. Cells exposed to PLO[a] during adsorption of virus	57	
D. Control, no PLO	23	C/D = 2.48
E. PLO[a] added after adsorption period[c]	42	
F. No PLO	31	E/F = 1.35

[a] PLO, 1.0 μg/ml, applied for 1 hr.
[b] Cells washed twice with diluent before challenged with VSV.
[c] Cells with adsorbed virus washed twice before and after exposure to PLO.

resistance to virus infection following uptake of interferon (5). The physical systems measure total uptake without regard to the potential biological activity that results. On the other hand, the bilogical systems, while measuring specifically the attachment of biologically effective stustances, fail to record the concomitant attachment of particles which are not detected by the biological systems employed. Systems which utilize biological detection necessarily are limited to concentration of polycations that are not grossly toxic to cells, whereas physical systems impose no such limitation.

A thorough analysis of the various types of polycation-induced uptake was presented by Ryser (16). He noted that with concentrations of polycation that are not toxic to cells, there is enhancement of the uptake of labeled albumin by cells of Sarcoma 180 and postulated the mechanism to be an increase in pinocytosis induced by membrane distortion of attached polycation. With higher concentrations of polycation which resulted in some loss of dye exclusion, and presumably in reversible toxicity, there was greater uptake of labeled albumin and also biologically detectable uptake of infectious viral RNA and interferon (2, 5). The mechanism whereby this was effected, according to Ryser (16), is an increase in permeability of the cell membrane as evidenced by the demonstration of membrane defects and toxic vacuoles in electron micrographs. Finally with very high concentrations of polycation, which result in complete disruption of cell membranes, there was a much greater uptake of labeled albumin. This uptake was ascribed to the availability of previously inaccessible intracellular sites of attachment.

The enhancement of VSV titer noted in the present study occurred at a concentration of PLO that is not associated with detectable cell toxicity. It occurred with both CEF and MSF and therefore appears to lack cell specificity. On the other hand, at least 6 other viruses studied thus far were not enhanced at this concentration of PLO, indicating a *virus* specificity (17). Since pretreatment of cells but not of virus resulted in enhancement, it appears that PLO can effect a change in cells that leads to enhancement of virus uptake. Furthermore, it is probably the attachment phase of uptake that is enhanced since the effect can be demonstrated at 4° at which temperature the later steps of infection are not expected to occur. This evidence is supported by the kinetic data revealing a marked enhancing effect as early as 5 sec after onset of the adsorption period. Thus, PLO appears to affect cell membranes in some manner which permits enhanced attachment of certain viruses.

Although enhancement of VSV titer occurs at a concentration similar to the first concentration range of Ryser (16), it is apparently not due to an increase in pinocytosis. The latter phenomenon would be expected not to be virus specific and since it requires energy, it should be markedly retarded at 4°. With the evidence that cell membranes have an isoelectric point of 2.2 (18) and therefore a grossly negative charge at physiological pH, one of 2 other possibilities seems likely: either the polycation could act as a new receptor, combining with the cells at one end and with the virus elsewhere, or the polycation, by neutralizing the charge at the membrane surface, could make available cellular sites that otherwise are inaccessible.

There is some evidence against the hypothesis that the polycations themselves act as receptors. (i) Pretreatment of virus would be expected to produce enhancement by the same mechanism, but such enhancement does not occur. (ii) Such a gross mechanism would not be expected to be virus specific. (iii) The hypothesis does not explain the decrease in VSV enhancement with concentrations of PLO greater than 1.0 μg/ml and not toxic for cells (Fig. 1). On the other hand, these objections do not militate against the hypothesis that new cell receptor sites are exposed by the neutralization of the superficial charge on the cell surface. The sharp rise and fall of VSV enhancement over a relatively narrow range of concentration around 1.0 μg/ml of PLO may represent a stoichiometric titration of a charged grouping. That only certain viruses can take ad-

vantage of the new constellation of charges is acceptable. Finally, the possible trimming of the surface charge with 1.0 μg/ml of heparin simultaneous with PLO leaving to even greater enhancement (Table I) is provacative. Since the evidence against the first hypothesis and for the second is also open to other interpretations, some reservations must still be held as to the specific nature of the change induced in cell membranes by PLO that leads to enhanced uptake of VSV.

It is interesting that the kinetic data showed the virus titer to vary in a straight-line relationship with the square root of time only when PLO was present. This is consistent with attachment occurring as a function of the collision frequency due to Brownian motion as calculated by Valentine and Allison (19) from the theory of Crank (20). The fact that this was not apparently true for the control raises the question, "Is the enhancibility of VSV virus due to otherwise poor attachment because of a relatively defective complement of combining sites?". This possibility is currently under investigation.

Summary. The presence of 1.0 μg/ml PLO during the adsorption period increased the resulting plaque numbers of VSV severalfold over that of PLO free controls. The enhancement was demonstrated on both CEF and MSF in the absence of detectable cell toxicity, occurred at both 4 and 37°, was manifest with as little as a 5-sec adsorption period, and could be completely inhibited by the simultaneous presence of heparin. Treatment of cells alone, but not virus alone, prior to the adsorption period, resulted in similar but less enhancement in the number of plaques. It appears that 1 μg/ml of PLO alters a cell monolayer so that certain viruses will have an enhanced rate of attachment.

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