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The Effect of Polycations on Cell Membrane Stability and Transport Processes

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Summary. The interaction of poly-L-lysines of different molecular weights (PL) with Ehrlich ascites tumor cells was studied experimentally with respect to cell surface binding, cell electrophoresis, cytotoxicity and membrane permeability. Although they decrease the net negative charge of Ehrlich ascites cells similarly at low PL concentrations, low molecular weight PL was less cytotoxic and less damaging to the potassium transport mechanism than was high molecular weight PL. At certain PL concentrations, membrane damage was reversible on reincubation in PL-free media. The amount of bound polylysine as determined with fluorescent labeled polylysine was compared by electrophoresis to the amount of polylysine expressed on the electrokinetic surface. The results indicated that only a small fraction of polylysine bound to Ehrlich ascites tumor cells was electrokinetically detectable. The adsorption of polylysine to Ehrlich ascites tumor cells was not describable by the usual adsorption isotherms. It is suggested that the same number of monomeric lysine units of high and low molecular weight PL are adsorbed at the cell electrokinetic surface, but cytotoxicity is dependent on molecular weight. Although the negative charge of human red blood cells could be reversed at low PL concentrations, no such effect could be observed for ELD (a subline of Ehrlich ascites carcinoma) cells even at high PL concentrations. The relationship of PL binding to the stimulation of macromolecular uptake is discussed.

It is known that polycations may bind to the surfaces of animal cells [16, 22] and may be cytotoxic (D. Cormack, *personal communication*). Furthermore, it has been shown that polycations such as polylysine and DEAE-dextran may promote the uptake of proteins [20], and single- and double-stranded polynucleotides [4, 7, 14] into animal cells. This action of polycations has already proven useful in the process of inducing interferon production [4], and may, in the future, prove useful in the treatment of genetically defective cells with exogenous nucleic acids [6].

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The present communication describes a detailed experimental treatment of the action of flexible polycations on cell surfaces. We specifically treat the interaction of poly-L-lysine with the surfaces of Ehrlich ascites tumor cells; however, we believe that the conclusions presented here are applicable to a variety of combinations of flexible polyelectrolytes and animal cells. The binding of polylysine to the cell surface has been measured by cell electrophoresis, and uptake of fluorescent labeled poly-L-lysine and the resulting changes in membrane integrity as measured by dye exclusion and by K^+ efflux have been investigated.

Materials and Methods

Cells

Cells of the Ehrlich-Lettré hyperdiploid subline of Ehrlich ascites carcinoma (ELD) were grown in suspension culture or in mice as described previously [11]. Cells were prepared for experiments by washing three times in RPMI medium 1630 [17] and finally resuspending at concentrations ranging from 1 to 20×10^6/ml in RPMI 1630. Cell counts were made using a haemocytometer or a Coulter Counter, model B. In some experiments, fresh, washed, human red blood cells (RBC) suspended in isotonic buffer were used.

Enzyme Treatment

ELD cells were washed twice in Hanks balanced salt solutions (HBSS) and resuspended in HBSS at 37 °C at a final concentration of 10^5/ml. Aliquots of this suspension were added to neuraminidase (*Vibrio cholerae* strain 4, General Biochemicals Corp.) to give a final concentration of 1×10^6 cells and 10 units neuraminidase/ml. The cell suspensions were incubated at 37 °C for 30 min, washed twice and finally resuspended in RPMI 1630.

Polylysine (PL) Treatment

Washed ELD cells were added to RPMI 1630 containing different concentrations of poly-L-lysine to give a final concentration of 0.5 to 6×10^6 cells/ml. Four types of polylysine-hydrobromide or hydrochloride were used: (a) Mean mol wt = 170,000 (Schwarz-Mann) (HCl); (b) mol wt = 120,000 (Schwarz-Mann) (HCl); (c) mol wt = 16,000 (Mann Research Laboratories) (HCl); (d) mol wt = 2,800 (Sigma Chemical Corp.) (HBr). The calculated degrees of polymerization (DP) of these compounds are (a) DP = 813, (b) DP = 574, (c) DP = 78 and (d) DP = 13. The cell suspensions were incubated for 1 hr and at this time cellular parameters such as electrophoretic mobility, cation content and viability were determined.

Fluorescein Labeled Poly-L-lysine

Fluorescein isothiocyanate was coupled to PL of mol wt 2,800, 16,400 and 120,000 by standard procedures [2]. Subsequently, the complexes were purified through Sephadex G-25 columns. The PL concentrations in the complexes were determined by means

of the Lowry assay [11]. The ratio of fluorescein isothiocyanate (FITC) to lysine monomers was adjusted so that no more than 1 in 20 lysine residues were coupled assuming all the FITC present was complexed to PL. In this way the number of positive charges per PL molecule was not significantly decreased.

Binding of FITC-PL to Cells

ELD cells from culture, previously washed with PBS were incubated in a medium containing 0.145 M NaCl, 0.006 M KCl adjusted to pH 7.2 with 0.01 M phosphate buffer. The final FITC-PL and cell concentrations were varied depending on the experiment. The cells with FITC-PL were incubated at 37 °C for 30 min and then centrifuged at $500 \times g$ for 10 min. The supernatants were decanted off the cells and the fluorescence was measured using an Eppendorf fluorimeter with a λ 366 cut-off filter in the source-beam [2]. The FITC-PL concentration of the supernatant was determined using a series of FITC-PL solutions as standards. The fluorescences of supernatants of cells treated with unlabeled PL at each concentration were used as control for background fluorescence. The difference between initial FITC-PL concentrations and that determined by fluorescence was the amount of FITC-PL cellularly bound.

Cell Viability

Cell viability was assessed by adding one volume of 0.1% trypan blue in HBSS to one volume of cell suspension at 1 to 1.5×10^6 cells/ml and counting the number of stained ("dead") and unstained ("live") cells using a haemocytometer. Counts were usually made within 15 min of mixing cells with trypan blue indicator.

Intracellular Cation Content

Cell suspensions containing 1.0 to 1.5×10^6 cells/ml and various concentrations of polylysine were washed rapidly three times in iso-osmotic choline chloride at 4 °C and resuspended in 10 ml distilled water to a final concentration of 1.0 to 1.5×10^7 cells/ml. The sodium and potassium contents of the solutions were determined using a flame photometer as described previously [13]. In some experiments cells were washed in RPMI 1630 after polymer treatment, resuspended in RPMI 1630 plus 5% calf serum and incubated at 37 °C for 1 hr. The cells were then washed and prepared for flame photometry as described above. The standard deviation of cellular cation content determination was approximately $\pm 5\%$ of the mean.

Cell Electrophoresis

The electrophoretic mobilities of cells suspended in control or PL-containing media were measured at 37 ± 0.5 °C in a cylindrical tube apparatus with sintered grey platinum electrodes as described previously [1, 12]. In most instances the mobilities of 50 or more cells were determined for each sample and in most experiments the electrophoretic and viability determinations were made on coded samples.

Cell Volumes

In one series of experiments the packed cell volumes of cells treated with different concentrations of PL were determined using a micro-capillary centrifuge. The standard deviation of cell volume determinations was approximately $\pm 12\%$ of the mean.

Results

Cell Electrophoresis

Measurements of cellular electrophoretic mobility provide a useful index of the binding of polyelectrolytes at the electrokinetic surface of cells [16, 19, 21]. The electrophoretic results are plotted as a percentage of the control since the control mobility varied from -1.37 to -1.77 μ sec^{-1} V^{-1} cm in different experiments. This variation was due to metabolic and growth related factors as described in previous communications [12, 15].

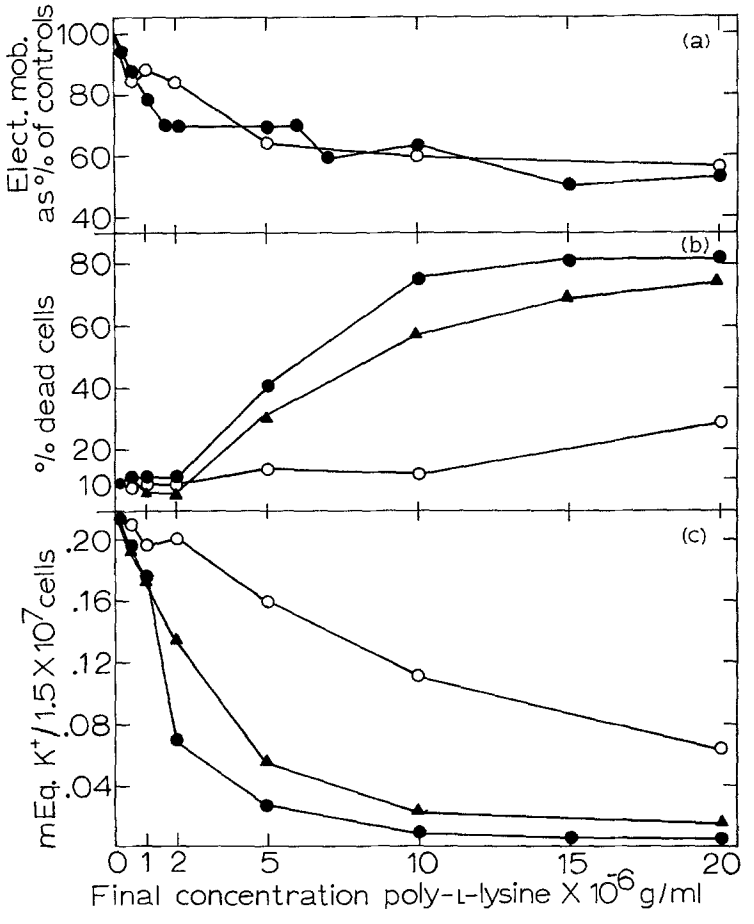

Fig. 1. This figure illustrates changes in (*a*) electrophoretic mobility, (*b*) cell viability as measured by dye exclusion, and (*c*) K^+ content: resulting from one hour's exposure at 37 °C to various concentrations of poly-L-lysine (PL): •—• 170,000 mol wt; ▲—▲ 16,600 mol wt; ○—○ 2,800 mol wt. Each point of the electrophoresis data represents the mean of at least 50 determinations from two to five separate experiments while each point of the viability and K^+ content data represents the mean of at least three determinations, also in two to five separate experiments

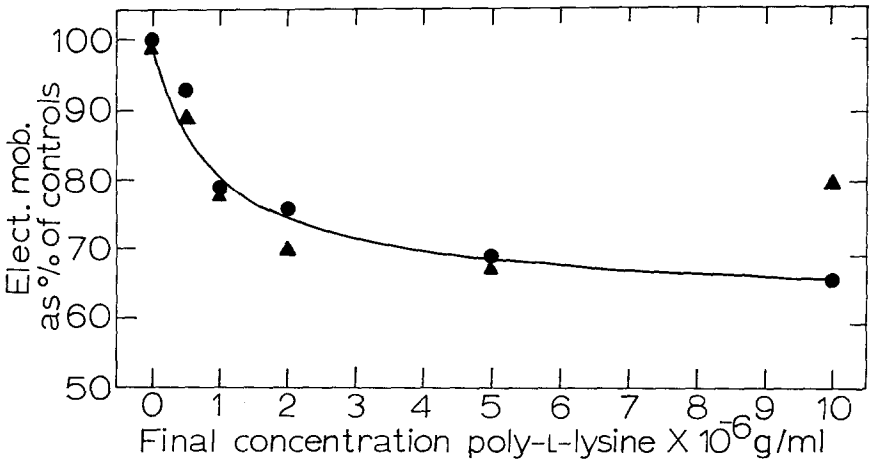

Fig. 2. Action of poly-L-lysine on the electrophoretic mobility of neuraminidase-treated cells and control cells. Cells were either treated with neuraminidase as described in the text, or maintained in culture medium. Subsequently, both samples were exposed to various concentrations of poly-L-lysine (170,000 mol wt) and the mobilities were measured. •—• control cells; ▲—▲ neuraminidase-treated cells. Each point represents the mean of at least 50 cells

The net negative cellular electrophoretic mobility was sharply reduced with increasing PL concentrations up to approximately 2 μg/ml PL (Fig. 1*a*). From 2 μg/ml to approximately 20 μg/ml with both high and low molecular weight PL, a plateau is evident with the mobility decreasing gradually. At higher PL concentrations not shown in Fig. 1, the low and high molecular weight PL differed in their effects on electrophoretic mobility. Above 50 μg/ml PL cells treated with the high molecular weight material exhibited a positive electrophoretic mobility while cells treated with low molecular weight material maintained a negative mobility. We were not able to induce ELD cells treated with 2,800 mol wt PL to undergo charge reversal.

Neuraminidase reduced the mean mobility of ELD cells from -1.53 to -1.13 μ sec^{-1} V^{-1} cm. The electrophoretic mobilities of neuraminidase-treated cells and control cells, both treated with 170,000 mol wt PL gave similar curves when the values at each PL concentration were plotted as a percentage of the mobilities in the absence of PL (Fig. 2). This result suggests that PL may bind equally well to sialic acid and to other anionic sites.

Cell Viability

In this report cell death was measured by the entry of trypan blue into the cell interior. The results are shown in Fig. 1*b*. Detectable cell death

occurred at 5 μg/ml for 170,000 mol wt PL. Most cells were dead at 10 μg/ml. Cell death was not observable at less than 20 μg/ml for 2,800 mol wt PL. At higher doses of 2,800 mol wt PL, cell death gradually increased but more than 50% of cells were viable until at least 50 μg/ml PL was reached. PL of mol wt 16,400 had effects on cell viability intermediate to those of the other molecular weights.

Intracellular Potassium Content (Fig. 1c)

Cells lost some K^+ at even low concentrations of 170,000 mol wt PL, although the losses at 1 μg/ml or less were slight. Between 2 and 5 μg/ml, K^+ loss was extensive. At 5 μg/ml and higher, the intracellular K^+ concentration was approximately equivalent to that of the extracellular medium. The loss of K^+ was much less with 2,800 mol wt PL; even at 10 μg/ml the cells had approximately 50% of their normal potassium content. PL of mol wt 16,400 had effects intermediate to those of the other molecular weights, although as Fig. 1*c* indicates the effects were more similar to those shown by the 170,000 mol wt PL than those of the 2,800 mol wt PL.

Reversibility of the Intracellular K^+ Loss

Table 1 shows that the intracellular K^+ concentration of control cells and cells previously treated with 1 μg/ml 170,000 mol wt PL, where there was little potassium loss, did not change significantly on reincubation in PL-free media.

In cells treated with 2 μg/ml 170,000 mol wt PL or 10 μg/ml 2,800 mol wt PL the intracellular K^+ was reduced but it recovered towards the control

Table 1. Recovery of intracellular K^+ by ELD cells after removal of extracellular poly-L-lysine by washing in PL-free media

Treatment	Mol wt of poly-L-lysine $\times 10^3$	Final conc. poly-L-lysine (g/ml)	mEquiv K/1.5 $\times 10^7$ cells [a]		Change in mEquiv K in 1 hr
			After treatment with poly-L-lysine	After resuspension in growth medium for 1 hr	
Controls	–	–	0.218	0.204	−0.014
Poly-L-lysine	170	1	0.191	0.185	−0.006
Poly-L-lysine	170	2	0.116	0.167	+0.051
Poly-L-lysine	170	5	0.037	0.035	−0.002
Poly-L-lysine	2.8	10	0.135	0.185	+0.060

[a] ± SEM.

level when the cells were placed in PL-free media. No such recovery was detectable for cells previously incubated with 5 μg/ml of 170,000 mol wt PL.

These results indicate that treatment with selected amounts of PL can result in major changes in cell membrane permeability, as measured by K^+ loss, without leading to irreversible cell damage.

Comparison of ELD Cells and Red Blood Cells

In one series of experiments the electrophoretic mobilities of human red blood cells (RBC) and ELD cells in the presence of 2,800 mol wt PL were

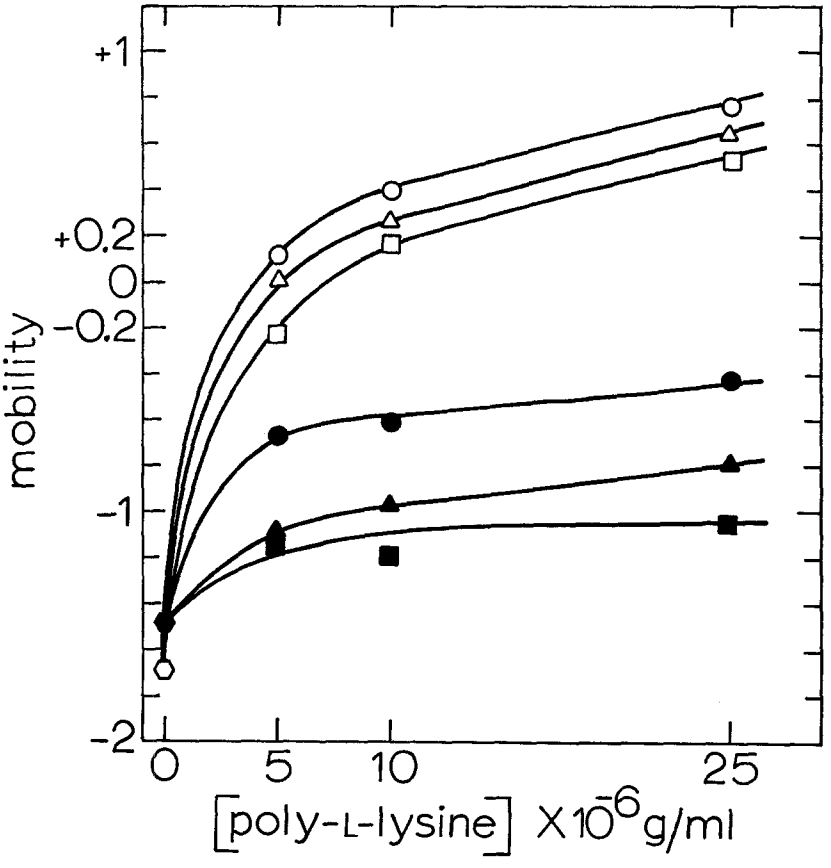

Fig. 3. The electrophoretic mobilities of red blood cells and ELD cells treated with poly-L-lysine. Cells of both types were washed and suspended in an isotonic buffer, pH 7.2 containing various amounts of PL of mol wt 2,800. After a 1-hr incubation at 37 °C the cellular electrophoretic mobilities were measured. An average of 10 cells per point were measured. The cell numbers were as follows: (1) red cells: ○—○ 5×10^5/ml; △—△ 4×10^6/ml; □—□ 2×10^7/ml. (2) ELD cells: ●—● 1.4×10^5/ml; ▲—▲ 1.1×10^6/ml; ■—■ 5.7×10^6/ml. The units of the ordinate are in $\mu\,\text{sec}^{-1}\,\text{V}^{-1}\,\text{cm}$

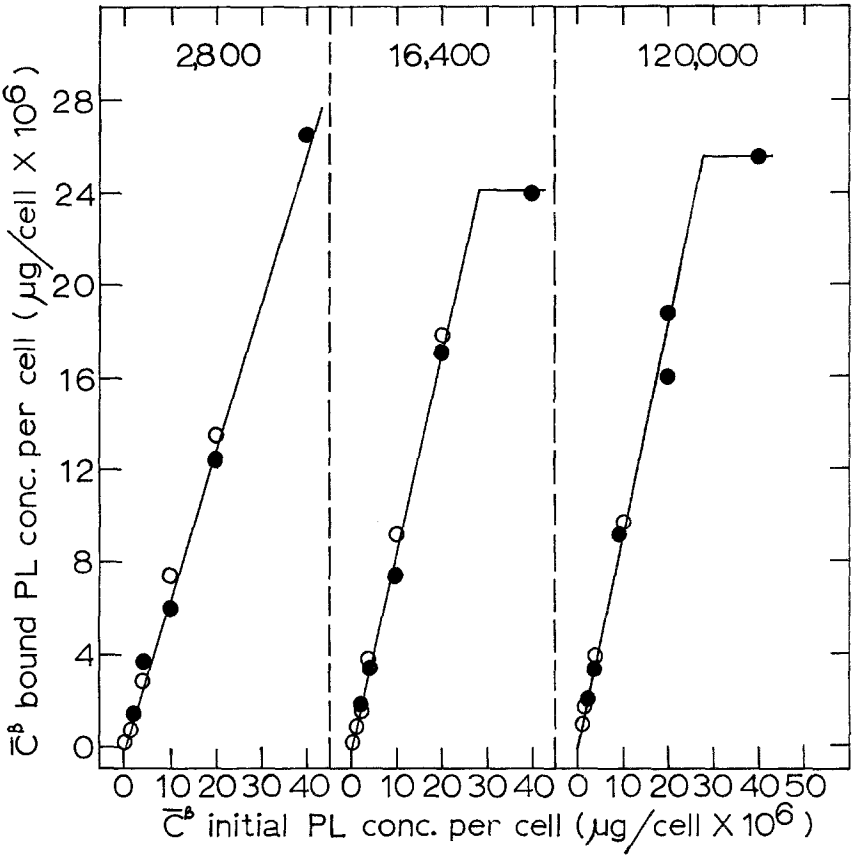

Fig. 4. Bound concentration polylysine per cell *vs.* initial polylysine concentration per cell for PL of mol wt 2,800, 16,400 and 120,000. The cell concentrations were: ○ 5×10^6 cells/ml; • 5×10^5 cells/ml. The plateaus for the 16,000 and 120,000 mol wt samples are supported by additional data points not shown in the figure for lack of space

compared. The cell concentrations were chosen such that the apparent surface areas of the different cell types per unit volume of solution were the same.

In these experiments both the cell concentration and the PL concentration were varied. The results are shown in Fig. 3. As can be seen, red cells and ELD cells behaved quite differently. Charge reversal occurred at 5 to 10 μg/ml PL for RBC whereas no charge reversal occurred for ELD cells. The behavior of RBC exposed to PL of mol wt 2,800, where the degree of polymerization equals 13, was quite similar to that reported by Nevo *et al.* [18] who used a PL of DP equal to 36. Furthermore, no hemolysis was observed by us upon treatment of RBC with PL of mol wt 2,800.

Binding of FITC-Labeled PL

Fig. 4 indicates that the amount of PL bound per cell was linearly related to the initial concentration of PL per cell up to an initial concentration of approximately 25 to 40 μg/10^6 cells depending on molecular weight. Within the limits of experimental error the amount of PL bound per cell was independent of initial concentrations of cells for equal initial PL concentration per cell. Somewhat more PL was bound with increasing molecular weight, but for the molecular weight PL studied most of the PL initially present in solution was absorbed to the cells. The actual amounts were mol wt 2,800 ~68%, mol wt 16,400 ~86% and mol wt 120,000 ~95%.

Fluorescence Microscopy

Fig. 5 shows the characteristic pattern of fluorescence of ELD cells after treatment with 120,000 mol wt FITC-PL. It can be seen that the fluorescence is concentrated in small patches on the surfaces of the cells and that no intracellular fluorescence is observed. The appearance of fluorescence

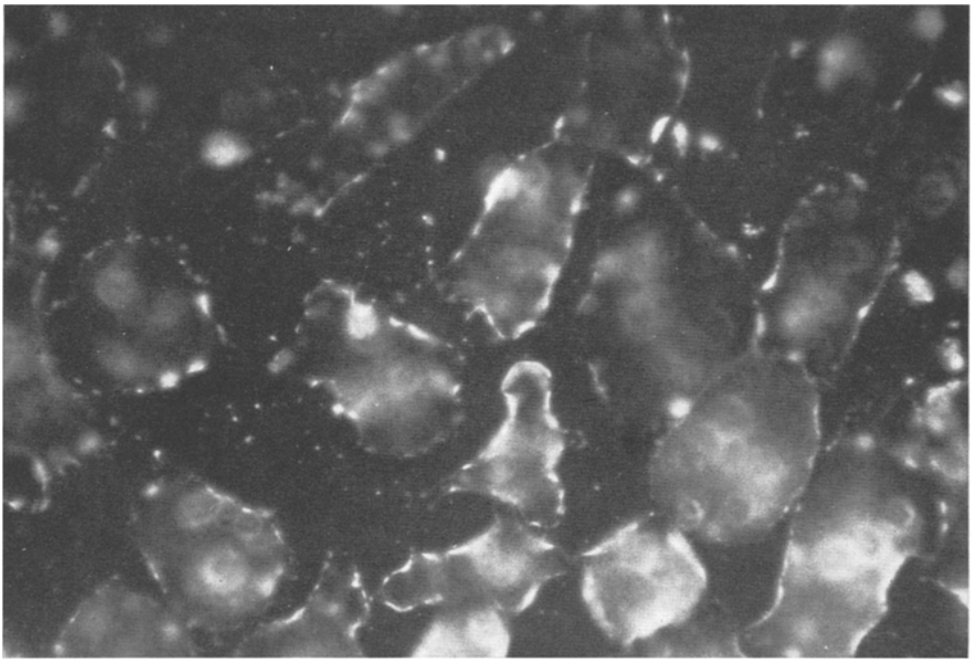

Fig. 5. Fluorescence of ELD cells (grown on glass) treated with FITC-labeled PL of mol wt 120,000 (final PL conc. 50 μg/ml). Scale on print 22 mm=20 μM or magnification=1,100×

Table 2. Volumes of ELD cells after treatment with different molecular weight polylysines

Treatment	Final concentration (μg/ml)	Vol/cell ($\mu^3 \times 10^{-3}$)[a]
Control	0	1.23
PL 2,800	5	0.88
	10	0.79
PL 16,400	5	1.18
	10	1.16
PL 120,000	5	1.22
	10	1.39

[a] Error is ±12% of the mean.

binding could be observed by flowing the FITC-PL across the cells. It appeared that the patches are found immediately after adding the FITC-PL and do not change position on the cell.

Cell Volume

Table 2 shows that in the presence of low molecular weight polylysine ELD cells had a somewhat smaller volume than in the control cells. The two higher molecular weight PL's were without detectable effects at the concentrations used.

Discussion

The electrophoretic mobilities of charged particles reflect the charge densities at the electrokinetic surface [23]. Thus, if the variation of mobility with concentration of polylysine is due to direct neutralization of anionic charge and/or the addition of cationic charge from the polylysine to the electrokinetic surface, an estimate of the number of positive charges so involved may be obtained by use of the Helmholtz-Smoluchowski equation (*see* [23]). In Table 3 the number of positive charges expressed at the electrokinetic surface has been calculated for two molecular weights: mol wt 170,000 and mol wt 2,800. The ratio of the number of positive charges detected for the high molecular weight material to those for the low molecular weight material suggests, to a first approximation, that the amount of expressed charge is independent of molecular weight. However, difficulties such as determining the position of the electrokinetic surface, the degree of ionization of the polylysine and the distribution of polylysine

Table 3. Number of detectable positive charges at the cell electrokinetic surface after incubation with poly-L-lysine

PL concentration (μg/ml)	Number of positive charges/cell		$R=\frac{n \text{ mol wt } (170{,}000)}{n \text{ mol wt } (2{,}800)}$
	PL mol wt = 170,000	PL mol wt = 2,800	
0	0	0	
0.5	4.7×10^6	9.7×10^6	0.48
1	14.4	8.5	1.70
2	16.5	11.8	1.39
5	21.1	25.8	0.82
10	22.8	28.3	0.81

prevent the direct electrokinetic determination of the amount of polylysine absorbed to the cells.

A more direct measure of polylysine absorbed to ELD cells is obtained for the FITC-PL measurements of PL binding. It is easily seen from Fig. 4 that the amount of bound polylysine is linearly related to the initial concentration of polylysine up to a limiting concentration. It is interesting to note that at the high concentrations indicated by this limit the cells are visibly morphologically altered. This proportional relationship was also indicated in studies on ELD cells with PL done by Kornguth, Stahmann and Anderson [10]. It is noted that this linear dependence which may be expressed by the relation

$$\bar{C}^b_{PL}=k\bar{C}^0_{PL}, \tag{1}$$

where $\bar{C}^b_{PL}$ is the amount of bound PL per cell and $\bar{C}^0_{PL}$ is the initial solution concentration of PL per cell, is not apparently consistent with a conventional absorption isotherm. In the latter case one expects relation of the form

$$\bar{C}^b_{PL}=f(C_{PL}) \tag{2}$$

where $f(C_{PL})$ is some function of the equilibrium concentration of PL. RBC as studied by Nevo *et al.* [18] differ from ELD cells in that RBC do follow an absorption isotherm. This point is discussed more fully in the Appendix to this paper.

Given the amount of polylysine absorbed per cell as determined by FITC-PL measurements one can compare the amount of positive charge that should be present at the cell surface (if each PL molecule carried its full charge) with that electrophoretically detected. This has been done

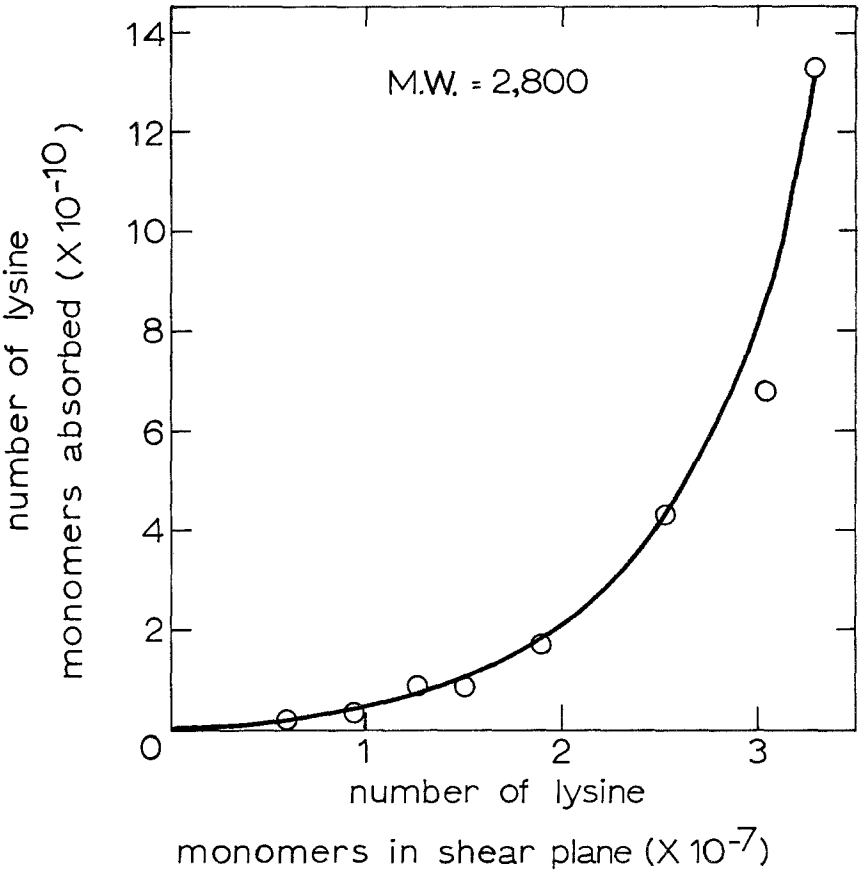

Fig. 6. The relationship between total absorbed polylysine and polylysine expressed at the electrokinetic surface

in Fig. 6 for PL of mol wt 2,800. It is apparent from this figure that only a small fraction of the total positive charge possible is expressed at the electrokinetic surface. Furthermore, the amount of charge expressed at the electrokinetic surface reaches a limiting value.

It seems possible that the cell-bound FITC-PL resides mainly on the cell membrane since fluorescence microscopic studies suggest that FITC-PL in the doses used here, fails to enter ELD cells.

Microscopic studies of FITC-PL absorbed to ELD cells indicated that the PL tends to aggregate into high density patches. However, since the electrostatic repulsions that would be created by packing the required number of charged polylysine into the relatively small patches would render that configuration energetically unfavorable, it must be concluded that the

polylysine molecules are effectively neutralized [9]. There is, however, no direct evidence of the identity of the neutralizing material.

It is apparent that the absorption of polylysine to ELD cells does profoundly alter the permeability of the cell membrane. This is indicated in the work of Kornguth and Stahmann [9] and in the present study. The results (Fig. 1*c*) show that polylysine decreases the amount of intracellular potassium. The extent of this effect is seen to be dependent on the PL concentration and the molecular weight rather than on the number of monomeric lysine units absorbed. The change in cellular K^+ level is apparently due to K^+ leakage rather than to an alteration of cell volume.

A similar dependence on molecular weight is obtained for the cytotoxicity of polylysine. These studies indicate that low molecular weight polylysine is much less toxic than PL of high molecular weight. It would again appear that the critical factor is not the number of monomeric lysine amount absorbed since equal amounts of absorbed polylysine of different molecular weights are not equally cytotoxic.

The response of red cells to PL treatment differed from that of cultured animal cells in terms of electrokinetic behavior (Fig. 3) and also in terms of membrane stability. While ELD cells and other cultured cells experience changes in membrane permeability and cell damage at high concentrations of PL (*see* Fig. 1 and references), red cells apparently remain intact. No hemolysis was observed by us upon treatment with PL of DP equal to 13, while Katchalsky *et al.* [8] reported that red cells, agglutinated with PL of DP equal to 200, may be restored to their original appearance by treatment with polyanions. Thus, poly-L-lysine could be used to explore differences between the surfaces of various cell types.

Our present results show that poly-L-lysine can alter the permeability of the ELD cell membrane at subtoxic levels. Such a disruption caused by PL or by other cationic polyelectrolytes, may have the following consequences: (a) the polyelectrolyte molecule itself may enter the cell; (b) substances which are normally retained within the cell may leak out; (c) substances in the extracellular medium which are normally excluded from the cell may enter. This may be related to the stimulation of macromolecular uptake mentioned earlier [4, 7, 14, 20].

Thus, polycations may promote the uptake of macromolecules by a process which is independent of cellular metabolism and which depends mainly on the nature of the cell surface and of the polycations. A process of this type would be quite distinct from the uptake of macromolecules by the mechanism of "pinocytosis" [3, 5].

Appendix

The Binding of PL for ELD Cells

The conservation of mass requires that the initial weight of polylysine added to solution m^0_{PL}, be equal to the equilibrium weight in solution m^e_{PL}, plus the weight of bound polylysine m^b_{PL}. Thus, we have

$$m^0_{PL} = m^e_{PL} + m^b_{PL}. \tag{A.1}$$

Furthermore, we note that

$$m^0_{PL}/N\,c = C^0_{PL}/C\,c = \bar{C}^0_{PL} \tag{A.2}$$

where Nc is the total number of cells in suspension and Cc is the number of cells per unit volume. Similar relations define $\bar{C}^b_{PL}$ and $\bar{C}^e_{PL}$. Therefore, another form of Eq. (A.1) is

$$\bar{C}^0_{PL} = \bar{C}^e_{PL} + \bar{C}^b_{PL}. \tag{A.3}$$

In general, adsorption from solution is fitted to an isotherm of the conventional form

$$\bar{C}^b = f(C^e) \tag{A.4}$$

where C^e is the equilibrium concentration and $f(C^e)$ represents some function of C^e. As a concrete example of this we use the Freundlich adsorption isotherm, which may be written as

$$\bar{C}^b_{PL} = K(C^e_{PL})^{1/n} \tag{A.5}$$

where n is an empirically determined number.

Thus, a Freundlich isotherm indicates that equal amounts of bound polylysine per cell would have the same equilibrium concentration. This expected behavior is not observed in the present experiments. For example, from Fig. 4 for mol wt of 2,800 at an initial concentration of 10 μg/cell we find that at the high cell concentration (open circles, cell concentration = 5×10^6 cell/ml) the amount of bound polylysine is 6.76×10^{-6} μg/cell. The low cell concentration (closed circles, $Cc = 5 \times 10^5$) has a bound weight of 6.04×10^{-6} μg/cell. However, the equilibrium concentration in the first case is found to be 16.18 μg/ml while in the second case it is 1.98 μg/ml. Therefore, it does not appear that the Freundlich equation fits this data, nor in fact will any relation of the form indicated by Eq. (A.4).

The data suggest that the form of the absorption relation should be:

$$\bar{C}^b_{PL} \propto f(\bar{C}^e_{PL}). \tag{A.6}$$

In fact, for concentrations below the plateau concentration we observe that the data in Fig. 4 is fitted by a relation of the form:

$$\bar{C}^b_{PL} = k\bar{C}^0_{PL}. \tag{A.7}$$

By Eq. (A.3) this suggests that the isotherm may be asymptotically given for low amounts of bound polylysine per cell by an isotherm of the form:

$$\bar{C}^b_{PL} = G\bar{C}^e_{PL}. \tag{A.8}$$

In the simplest case expressed by Eq. (A.6), G would have the value of $(k/1-k)$. We have not depicted the data in Fig. 4 using $\bar{C}^b_{PL}$ as a function of $\bar{C}^e_{PL}$ because we do not wish to imply that an adsorption isotherm for this case has been determined, but rather to suggest that the results indicate a divergence from normal solution adsorption.

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