Proc. Natl. Acad. Sci. USA
Vol. 76, No. 7, pp. 3246–3250, July 1979
Biochemistry

Antineoplastic activity of poly(L-lysine) with some ascites tumor cells

(cancer chemotherapy/polypeptides/Ehrlich ascites carcinoma/membrane perturbations/drug-carriers)

L. J. ARNOLD, JR., A. DAGAN, J. GUTHEIL, AND N. O. KAPLAN

Department of Chemistry, Q-058, School of Medicine, University of California at San Diego, La Jolla, California 92093

Contributed by Nathan O. Kaplan, April 23, 1979

ABSTRACT **We have found that poly(L-lysine) can be a very effective agent in preventing the growth of Ehrlich ascites tumors in mice. When given optimal doses of poly(L-lysine) (M_r 60×10^3) intraperitoneally for 5 consecutive days, beginning on day 1 after inoculation with Ehrlich ascites cells, White Swiss mice show nearly a 100% remission from subsequent tumor growth. Rechallenge of "cured" animals with tumor cells, however, shows no long-term immunological protection. In tissue culture, poly(L-lysine) shows a related potent cytotoxicity with HeLa cells; interestingly, the D isomer has properties strikingly different from those of the L isomer. In addition, there is a strong molecular weight dependence in that the small polylysine (M_r 3×10^3) possess less than 1/20th the cytotoxicity of large polymers (M_r 70×10^3) on a weight basis in both cell culture and animal studies. At the same time, none of these lysine polymers gives any significant increase in life span to BDF_1 mice infected with L_{1210} murine leukemia cells. We have also further explored the mechanism by which the polylysines express their cytotoxicity. These data indicate that lysine polymers show cell specificity in their action and in some cases they may be beneficial as potent antineoplastic agents, particularly when molecular weight is taken into consideration.**

For more than 25 years, poly(L-lysine) has been known to have unusual biological properties. Early studies showed that it decreased the infectivity of tobacco mosaic virus (1), disturbed thrombin formation in rats (2), blocked the development of bacteriophage (3, 4), protected chicken embryos from animal viruses (5, 6), and possessed antibacterial activity (7). There was also an early report that indicated that polylysine had some activity against murine tumors (8). More recently, polylysine has been found to exhibit a large number of unique membrane properties. These include the ability to enhance the cellular uptake of macromolecules (9), to inhibit iodide uptake by thyroid slices (10), to produce pathogenesis of the glomerular epithelium (11), to act as an anticholinesterase (12), to specifically agglutinate the lymphocytes from cancer patients (13), and to either increase or decrease the transport of specific radioisotopes into cells (14). These effects are probably associated with the polycationic character of polylysine and are probably due to specific interactions on the cell membrane.

Recently, we have been examining the ability of polylysine to serve as an efficient drug carrier (15). In doing the controls for these studies, we found that poly(L-lysine)s of a certain M_r, (60×10^3) could induce 100% remissions in mice inoculated with Ehrlich ascites when the polymer was administered at increased doses. This paper presents results on the antineoplastic activity and toxicity as functions of polymer molecular weight and concentration. Also, we will provide further information into the mechanism accounting for the antineoplastic and cytotoxic activities of polylysine.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Poly(L-lysine) hydrobromide (M_rs 3,000, 13,000, 35,000, 60,000, and 70,000) and poly(D-lysine) (M_rs 36,000 and 70,000) were purchased from Sigma and were used without further purification. Standard solutions of polylysine were prepared by weighing the dry lyophilized material and dissolving it in the necessary amount of phosphate-buffered saline or distilled water.

Animal Testing. Mice of the strain indicated (see figure and table legends) were inoculated intraperitoneally with 0.1 ml of either L_{1210} murine leukemia cells (10^5 cells per animal) or Ehrlich ascites carcinoma ($2–10 \times 10^6$ cells per animal) on day 0. Beginning on day 1, injections were given intraperitoneally for 5 consecutive days with the appropriate polylysine solution in approximately 0.1 ml. Subsequently, the animals were monitored for death as a result of tumor growth. Treated animals showing an increase in life-span of more than 300% in comparison to untreated controls were considered to be free of tumor due to treatment and the experiment was discontinued.

Toxicity Testing. Toxicity studies of polylysine in mice were carried out as indicated above with the exception that the mice received no tumor inoculation and they were subsequently monitored for death due to polylysine toxicity instead of tumor.

Cells and Culture. HeLa cells were maintained in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's media supplemented with 1.2 g of sodium bicarbonate per liter, 15 mM Hepes, 192 units of penicillin, 200 μg of streptomycin, and 25 μg of ampicillin per ml, 5% (vol/vol) horse serum, and 2.5% (vol/vol) fetal calf serum. This same medium was used in all experimental protocols. Stock cultures were grown on 100-mm plastic tissue culture dishes (Falcon) in a humidified atmosphere of 5% CO_2 in air at 37°C and were subcultured every 4–5 days.

For all experiments, subconfluent stock plates were aspirated, washed with phosphate-buffered saline ($Na/K/P_i$) (containing, per liter: 8 g of NaCl, 200 mg of KCl, 1.15 g of Na_2HPO_4, 200 mg of KH_2PO_4, and 15 mg of phenol red), and trypsinized with 0.1% trypsin/0.03% EDTA at 37°C. After the cells had detached (about 5 min) they were diluted with medium and an aliquot was counted in a model B Coulter Counter. A further dilution was made to achieve a final cell density of 10^4 cells per ml of medium; 2-ml aliquots of this solution were distributed to 35-mm tissue culture dishes (Falcon) and the plates were incubated overnight to allow cell attachment.

Cell Growth Assay. Experiments were started by replacing the medium with new medium containing polylysine at the appropriate concentration. At indicated times, duplicate plates were removed from the incubator, washed with $Na/K/P_i$ to

Abbreviation: $Na/K/P_i$, phosphate-buffered saline.

remove dead cells, trypsinized, and counted on a Coulter Counter.

Thymidine Incorporation Assay. Experiments were started by replacing the medium with medium containing [6-^{3}H]-thymidine (New England Nuclear) and an appropriate amount of polylysine. At the times indicated, duplicate plates were removed from the incubator, aspirated, and washed twice with Na/K/P_i. Free thymidine was extracted with cold 5% trichloroacetic acid for 15 min followed by a wash with the same solution. The remaining DNA and cell debris was then solubilized with 2 ml of 0.1% NaOH; 0.6 ml of this solution was taken for assay in 5 ml of Scintiverse (Fisher) with a Beckman model LS 230 scintillation counter.

RESULTS AND DISCUSSION

Poly(L-lysine) of M_r 60 × 10^3 showed a marked concentration-dependent cytotoxicity to HeLa cells in culture (Fig. 1*B*) as well as a dramatic antineoplastic effect against Ehrlich ascites carcinoma in White Swiss mice (Table 1). These results indicate that polylysine is cytotoxic, but it has some selective properties because at intermediate concentrations it can produce remission from Ehrlich ascites without producing gross toxicity. These results raise a question as to the degree of toxicity of polylysine at yet higher concentrations and the molecular weight dependence associated with toxicity. Fig. 1*A* shows the toxic effect of polylysine of different M_rs on the growth of HeLa cells in culture; Table 2 shows the approximate 50% lethal dose (LD_{50}) of polylysine of various M_rs in athymic mice. The higher M_r polymers were very toxic to animals and to HeLa cells in culture. In both systems, however, these effects were progressively decreased as the M_r of the polymer decreased. The fact that the smaller polymers had less activity in these cases suggests that there may be a similar correlation between M_r and the antineoplastic activity of polylysine as well. Table 3 shows the ability of polylysine at different M_rs, but at the same concentration, to act as an effective antineoplastic agent against Ehrlich ascites carcinoma in mice. It is clear from these data that there is also a striking correlation between M_r and antineoplastic activity.

It should be pointed out that this antineoplastic activity appears to be selective. In similar experiments with L_{1210} leukemia in BDF_1 mice (data not shown), no antineoplastic activity was observed; there was toxicity only at high doses of polylysine.

Table 1. Antineoplastic activity of poly(L-lysine) with increasing concentration

Poly(L-lysine), mg/kg	Mice, no.	Median life-span, days	Long-term survivors, no.
0	10	28	0
0.56	5	27	0
2.3	5	31	0
4.5	5	41	2
18.0	5	>90	5
36.0	5	>90	5

White Swiss mice were given 2 × 10^6 Ehrlich ascites cells intraperitoneally on day 0. Beginning on day 1, the mice were given M_r 60 × 10^3 poly(L-lysine) intraperitoneally in the amounts indicated, for 5 consecutive days.

These data raise the immediate question of the nature of both the toxicity and the antineoplastic activity of polylysine. In the case of the cytotoxicity with HeLa cells in culture, polylysine must be interacting directly with the HeLa cell. However, the antineoplastic activity could result from either a direct effect on the Ehrlich ascites cells themselves or indirectly by potentiating an immune mechanism responsible for suppressing the growth of Ehrlich ascites cells within the mouse. In order to test the likelihood of a long-term beneficial immune stimulation, we reinoculated mice that had previously survived Ehrlich ascites carcinoma by virtue of polylysine therapy. Six mice rechallenged with 4 × 10^6 Ehrlich ascites cells died with a median life-span of 22 days and five inoculated controls died with a median life-span of 24 days. These results indicate that no long-term immunological benefit is imparted by previous polylysine treatment. Because polylysine does not appear to promote any long-term immunologic benefit to these animals, the likelihood is increased that the antineoplastic activity arises from direct interaction between the polymer and the cancer cell. However, the possibility that polylysine facilitates a short-term immune response cannot be ruled out.

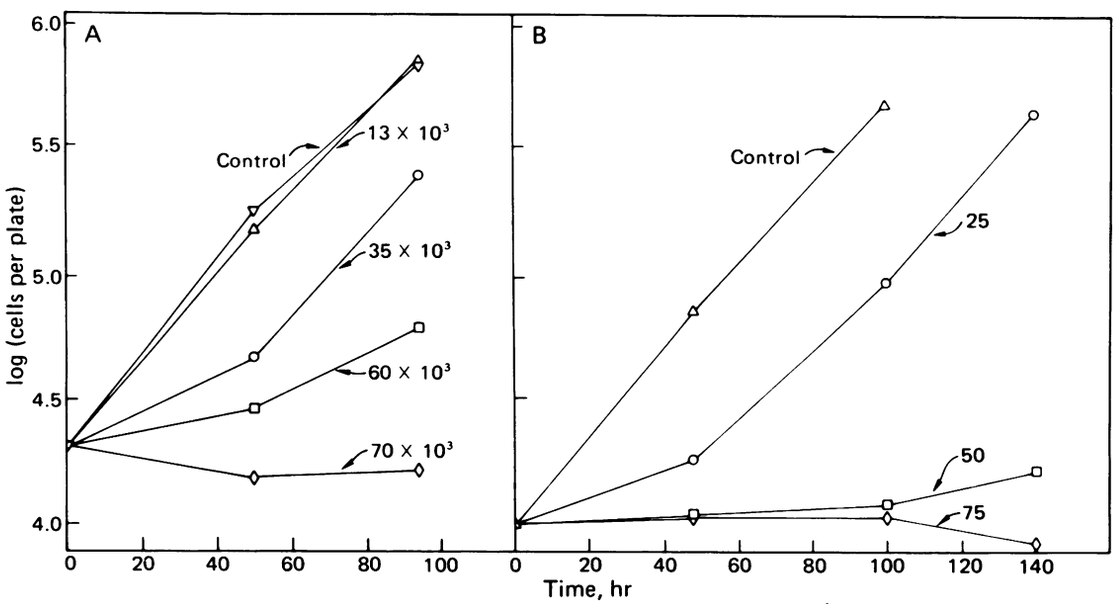

FIG. 1. Growth of HeLa cells. (*A*) As a function of poly(L-lysine) M_r at 25 μg/ml. Note that, with time, there was recovery of growth at intermediate M_r values. (*B*) In the presence of different concentrations (μg/ml) of M_r 35 × 10^3 poly(L-lysine). Note the growth after 48 hr with 25 μg/ml.

In order to determine the nature of the unique interactions between polylysine and transformed cells more clearly, we examined the relationship between polylysine and cell viability, as well as protein, RNA, and DNA synthesis. The relationship between the isomers of polylysine used and cell viability as determined by the uptake of [^{3}H]thymidine are shown in Figs. 2 and 3. Interestingly, over short periods, the L isomer was much more effective at inhibiting cell growth, as determined by thymidine incorporation, than was the D isomer. With time, however, these effects reversed themselves and the D isomer became more effective at inhibiting growth for long periods. The results suggest that, because of the opposing geometries of these molecules, they have differential abilities in inhibiting cell growth shortly after polylysine addition. With time, however, the L isomer is catabolized and the cells return to normal growth. In the case of the noncatabolizable D isomer, growth remains suppressed. In addition, the inhibition of RNA and protein synthesis parallels the inhibition of DNA synthesis. This implies that, at the time of polylysine addition, protein, DNA, and RNA synthesis (data not shown) can be completely inhibited [see, for example, poly(L-lysine) at 20 μg/ml in Fig. 2]. At the same time, they are inhibited simultaneously and to the same extent. It should be emphasized that at approximately 10 hr after treatment with poly(L-lysine) at 20 μg/ml these cells return to control growth.

In order to determine the number of cells that remained viable through poly(L-lysine) treatment, we carried out a 6-day cloning experiment. We were afraid that trypan blue exclusion would not give good results, because it was known that polylysine could promote the passage of small molecules across the membrane (16). Thus, trypan blue might indicate that the cells were dead when in fact they were not. Such cloning experiments showed that, with M_r 35 × 10^3 poly(L-lysine) at 20 μg/ml, 70–80% of the cells remained viable with respect to controls. It should be pointed out that these estimates are a conservative measurement of the number of cells viable after poly(L-lysine) treatment. This is due to the fact that the ratio of polylysine to cells in the cloning experiments was by necessity 100 times greater than in the thymidine uptake measurements. Moreover, as the polylysine per cells ratio goes up, we have found that polylysine cytotoxicity increases. The important point, however, is that with treatment with poly(L-lysine) at 20 μg/ml, a minimum 70% of the cells remained viable even though their DNA, RNA, and protein synthesis had been stopped for several hours. Cloning experiments showed no such recovery of viability when poly(D-lysine) was used.

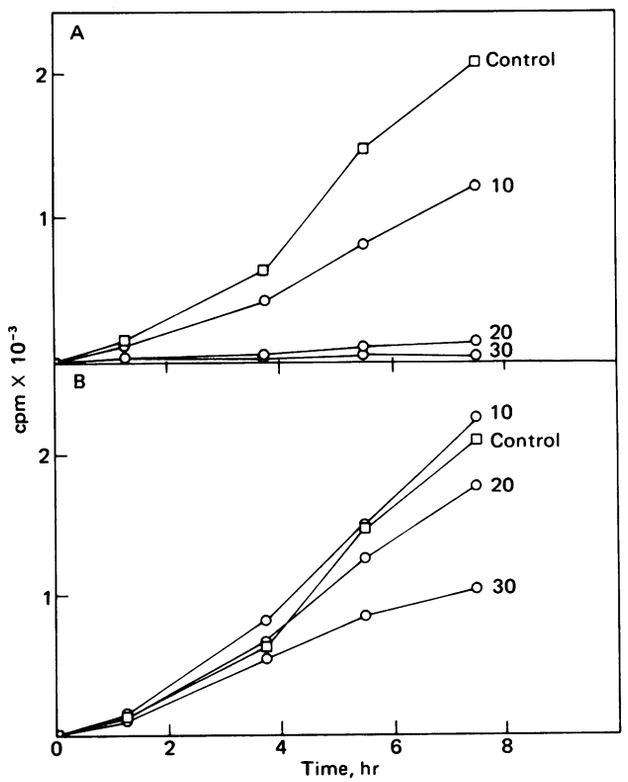

FIG. 2. Effects of increasing concentrations (μg/ml) of M_r 35 × 10^3 poly(L-lysine) (*A*) and M_r 36 × 10^3 poly(D-lysine) (*B*) on uptake of [6-^{3}H]thymidine into the DNA of HeLa cells in culture. At zero time, 10^4 cells in culture were treated with 26 pmol of [6-^{3}H]thymidine (0.6 μCi).

Table 2. 50% lethal dose of polylysines with increasing molecular weight

Compound and M_r	LD_{50}, mg/kg for 5 days
L-Lysine	>300
Poly(L-lysine), 3 × 10^3	>300
Poly(L-lysine), 13 × 10^3	100 ± 20
Poly(L-lysine), 35 × 10^3	60 ± 15
Poly(L-lysine), 70 × 10^3	30 ± 10
Poly(D-lysine), 70 × 10^3	<20

These toxicity studies were carried out with homozygous athymic BALB/c mice.

In addition to these results, we found that polylysine had a number of other effects on HeLa cells in culture. Histologically, at polylysine concentrations that did not greatly affect subsequent cell viability, the cells became enlarged and rounded and then showed an increased granulation. At higher concentrations of polylysine the cells were lysed and their components covered the surface of the plate as diffuse crenated discs. Under these conditions, the cells were not viable.

We also found that the effect of polylysine on cells in culture is inversely related to volume. This suggests that, at a fixed concentration of polylysine and number of plated cells, cell viability decreases as the volume of polylysine and medium above the cells increases. This observation indicates that polylysine has a high affinity for plated HeLa cells and that the larger volumes provide more polylysine for binding.

Evidence for high-affinity binding is also provided by our observation that approximately 80% of the short-term cytotoxic activity of polylysine is expressed even when the HeLa cells are washed thoroughly with fresh medium 15 min after treatment with polylysine. This suggests that, once bound, polylysine is not easily removed or that polylysine is capable of producing a membrane perturbation, the effects of which are not dependent upon its subsequent presence.

The present findings provide evidence that poly(L-lysine) has profound cytotoxic effects. In addition, with particular tumor types—namely, Ehrlich ascites carcinoma—polylysine is very effective in preventing tumor growth in mice.

The cytotoxic information we have obtained in culture is particularly interesting when compared to the previous data obtained by others. In this regard the striking concentration effects we observed for the toxicity of polylysine to HeLa cells in culture, the antineoplastic activity of polylysine with Ehrlich ascites tumors in mice, and the toxicity to mice correlate well with the concentration effects seen by others. Ryser (9) found that the ability of poly(L-lysine) and poly(D-lysine) to enhance the uptake of albumin into cells increased linearly with concentration. At the same time, Mayhew and coworkers (17) found that increasing molecular weights of polylysine increasingly promoted the leakage of potassium from Ehrlich ascites tumor cells.

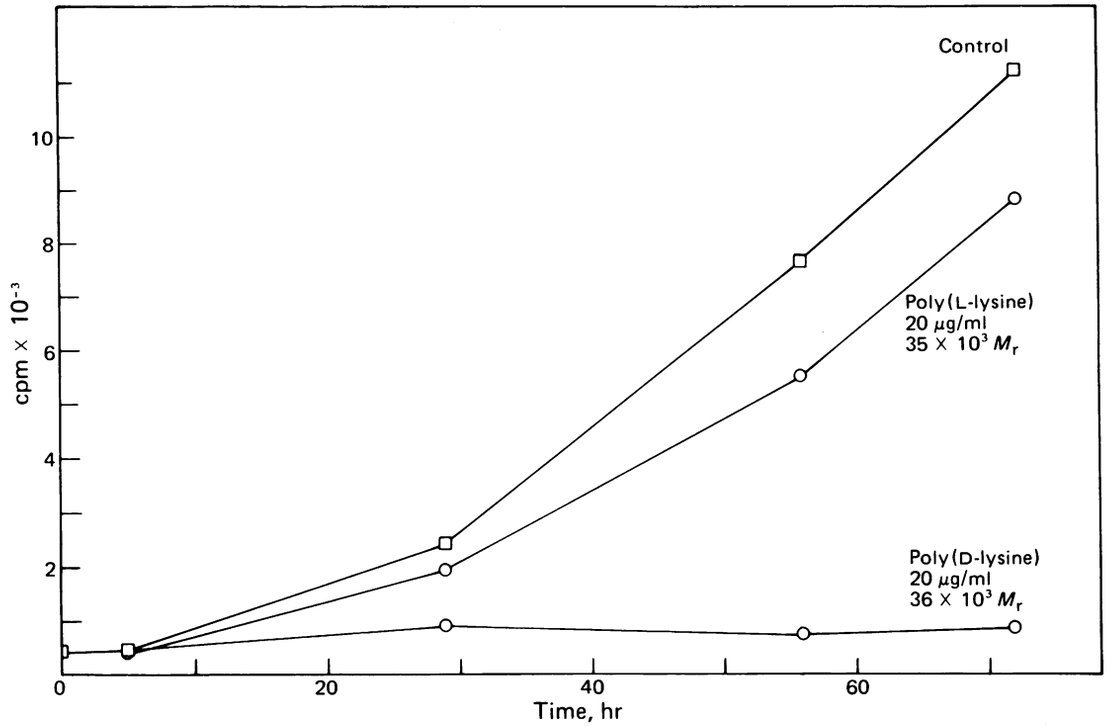

FIG. 3. Effect of polylysine on uptake of [6-^{3}H]thymidine into the DNA of HeLa cells over long periods of time; procedure as in Fig. 2 but only 0.2 μCi of [^{3}H]thymidine was added to each plate and it was added 24 hr before the polylysine. Note that over long periods of time, poly(D-lysine) inhibited cell growth much more than did poly(L-lysine), in contrast to the results in Fig. 2.

These effects appear to be primarily a result of an increased ability of large polymers to perturb cell surfaces and are not simply a result of increased binding. Studies conducted by Mayhew *et al.* (17) in which the binding of M_r 2800, 16,400, and 120,000 fluorescein-labeled polylysine was monitored showed that the different polymers were bound to a similar extent with increasing concentration.

The differential effects we see for the D and L isomers at short periods of incubation do not correlate with those found by Ryser (9). Our studies indicate that initially the L isomer is much more effective at preventing cell growth than is the D isomer. In Ryser's studies, on the other hand, the D isomer was an order of magnitude better than the L isomer at promoting the uptake of albumin in 2 hr. These results are not necessarily in disagreement, however, because the mechanism by which polylysine expresses its toxicity (or antineoplastic effect) may not be directly related to its ability to enhance the uptake of macromolecules.

Table 3. Antineoplastic activity of poly(L-lysine) with increasing molecular weight

Poly(L-lysine), M_r	Median survival*
None	18
3×10^3	17
13×10^3	39
35×10^3	60% (long-term)
70×10^3	80% (long-term)

The survival of heterozygous athymic BALB/c mice when given 25 mg of poly(L-lysine) per kg for 5 consecutive days beginning on day 1 after intraperitoneal inoculation with 10^7 Ehrlich ascites cells on day 0. Mice showing an increase in life-span of more than 300% were considered to be long-term survivors. Note that these animals were given 5 times as many tumor cells as those in Table 1.

* Median survival (days) or percentage long-term survivors if greater than 50%.

The ability of polylysine to inhibit the growth of cells is likely related to its ability to increase the efflux of small molecules from the cell which are required for DNA, RNA, and protein synthesis. For example, in addition to the increased efflux of potassium, it was found that polylysine promotes the leakage of inorganic phosphate, carbohydrates, free amino acids, small peptides, and adenosine 5′-monophosphate into the supernatant (16). Recently, the ability of a related polycation, poly(L-ornithine), to inhibit the growth of mouse L cells has been correlated in part to the leakage of potassium from the cells (18).

The very recent studies by Shier *et al.* (19) show that poly(L-lysine), as well as other polycations such as poly(L-ornithine) and poly(L-arginine), can activate membrane phospholipase A2 in 3T3-4a Swiss mouse fibroblasts. This finding provides an intriguing mechanism by which polylysine may affect membrane permeability to small molecules by promoting the hydrolysis of membrane phospholipid.

Curiously, Shier *et al.* did not think that this activity directly accounts for the cytoxicity because only 5 μg of M_r 15,000 poly(L-lysine) is required per ml for phospholipase A2 activation and 50 μg/ml is required for cytotoxicity. The results we present here—i.e., that low concentrations of poly(L-lysine) temporarily halt cell metabolism without greatly decreasing cell viability—suggest that a concentration of 5 μg/ml has temporary but not permanent effects on the cells. Only when large quantities of poly(L-lysine) are used, so that the polymer is not catabolically inactivated in a brief period of time, does the poly(L-lysine) become cytotoxic. Thus, we think that there is a good likelihood that persistent activation of phospholipase A2 by polylysine does contribute to its cytotoxicity.

In summary, our results, taken together with those of others, suggest the following model for the interaction of polylysine with cancer cells.

(*i*) Polylysine, either poly(L-lysine) or poly(D-lysine), binds

tightly to the cell membrane and induces morphological changes.

(*ii*) Associated with such binding is a rapid leakage of small molecules across the cell membrane, perhaps promoted by phospholipase A2 activation.

(*iii*) Also associated with polylysine binding is the rapid loss of RNA, DNA, and protein synthesis.

(*iv*) When poly(L-lysine) is used with HeLa cells in culture, it causes a greater degree of inhibition for short periods of time than does the D isomer.

(*v*) After approximately 10 hr, however, the L isomer is apparently degraded and the lesion corrects itself because the cells return to control growth.

(*vi*) In contrast, with increasing time, poly(D-lysine) shows increasing inhibition and, at long times (days), cells increase only slowly in growth, if at all. In this case, the lesion is presumably not corrected because the D isomer is not degraded.

(*vii*) The effects of the M_r and concentration of polylysine parallel each other in several systems including HeLa cell cytotoxicity in culture, depression of Ehrlich ascites tumor growth in mice, and toxicity to mice. In all cases the higher M_r polymers and concentrations have greatly enhanced effects.

(*viii*) Poly(L-lysine) has a potent activity with Ehrlich ascites tumors in mice but little effect upon L_{1210} tumors.

The fact that polylysine reacts much more potently with some tumors than others is probably a result of either a more specific interaction between polylysine and specific cells or a greater perturbation upon binding. The fact that polylysine is capable of producing a selective toxicity to some tumor cells, particularly in the ascites form, may be due in part to the greater negative charge on the surface of such cells (20). Consistent with this suggestion are the findings by Anghileri *et al.* (14) that ^{57}Co-labeled poly(L-lysine) is selectively bound to tumor tissues in comparison to the adjacent normal tissues. Other studies related to these findings show that copolymer poly(ornithine,leucine) is taken up selectively by different cell types (21), and it has been found that transformed cells are specifically aggregated by this polymer (22). However, polyanions such as dextran sulfate (23) show some related effects with Ehrlich ascites cells in culture, suggesting that features other than positive charge play a role in these activities. Resolution of all the factors responsible for these unique properties requires further studies with charged and uncharged polymers.

The potent effects of polylysine must be considered when it is used as a drug carrier. However, from studies conducted by Ryser and Shen with methotrexate-polylysine (24) and by us on methotrexate-polylysine (unpublished data) and 6-aminonicotinamide succinate polylysine (15), it is clear that polylysine has good potential as a drug carrier because drug conjugates with polylysine have more activity than does polylysine alone. Furthermore, from the present study it is likely that the low M_r polymers will be of particular value because they have limited toxicity.

We thank the National Institutes of Health (CA 11683) and the American Cancer Society (BC-60) for support. We also thank the National Cancer Institute (CA-23052-01) for support of the athymic mouse facility.

1. Stahmann, M. A., Graf, L. H., Patterson, E. L., Walker, J. C. & Watson, D. W. (1951) *J. Biol. Chem.* **189,** 45–52.
2. DeVries, A., Feldman, J. D., Stein, O., Stein, Y. & Katchalski, E. (1953) *Biol. Med.* **82,** 237–240.
3. Watson, D. W. & Bloom, W. L. (1952) *Proc. Soc. Exp. Biol. Med.* **81,** 29–33.
4. Shalitin, C. & Katchalski, E. (1962) *Arch. Biochem. Biophys.* **99,** 508–516.
5. Green, M. & Stahmann, M. A. (1953) *Proc. Soc. Exp. Biol. Med.* **83,** 852–858.
6. Rubini, J. R., Rasmussen, A. F. & Stahmann, M. A. (1951). *Proc. Soc. Exp. Biol. Med.* **76,** 662–665.
7. Bichowski-Slomnicki, L., Berger, A., Kurtz, J. & Katchalski, E. (1956) *Arch. Biochem. Biophys.* **65,** 400–413.
8. Richardson, T., Hodgett, J., Lindner, A. & Stahmann, M. A. (1959) *Proc. Soc. Exp. Biol. Med.* **101,** 382–386.
9. Ryser, H. J.-P. (1974) in *Rehovot Symposium on Peptides,* eds., Blout, E. R., Bovey, F. A., Goodman, M. & Lotan, N. (Wiley, New York), pp. 617–628.
10. Kawada, J., Yoshimura, Y. & Takeshi, M. (1976) *Endocrinol. Jpn.* **23,** 221–225.
11. Seiler, M. W., Rennke, H. G., Venkatachalam, M. A. & Cotran, R. S. (1977) *Lab. Invest.* **36,** 48–61.
12. Lin, S.-Y., Liao, C. & Lee, C. Y. (1977) *Biochem. J.* **161,** 229–232.
13. Ax, W., Schottler, S. & Bauer, H. W. (1978) *Br. J. Cancer* **37,** 319–321.
14. Anghileri, L. J., Heidbreder, M. & Mathes, R. (1976) *J. Nucl. Biol. Med.* **20,** 79–80.
15. Arnold, Jr., L. J., Dagan, A., Simon, S. D. & Kaplan, N. O. (1978) *Proc. Am. Assoc. Cancer Res.* **19,** 114, abstr. 454.
16. Kornguth, S. E. & Stahmann, M. A. (1961) *Cancer Res.* **21,** 907–913.
17. Mayhew, E., Harlos, J. P. & Juliano, R. L. (1973) *J. Membr. Biol.* **14,** 213–228.
18. Ogawa, K. & Ichihara, A. (1978) *J. Biochem.* **83,** 519–525.
19. Shier, W. T., Trotier, J. T. & Durkin, J. P. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 406, abstr. 937.
20. Ambrose, E. J., James, A. M. & Lowick, J. H. B. (1956) *Nature (London)* **177,** 576–577.
21. Duksin, D., Katchalski, K., E. & Sachs, L. (1975) *FEBS Lett.* **60,** 21–25.
22. Duksin, D., Katchalski, E. & Sachs, L. (1970) *Proc. Natl. Acad. Sci. USA* **67,** 185–192.
23. McCoy, G. D., Resch, R. C. & Racker, E. (1976) *Cancer Res.* **36,** 3339–3345.
24. Ryser, H. J.-P. & Shen, W.-C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3867–3870.