Effect of Polylysine on the Leakage and Retention of Compounds by Ehrlich Ascites Tumor Cells*

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SUMMARY

Washed Ehrlich ascites tumor cells were suspended in either a saline or a polylysine saline solution. The cells were centrifuged, and the supernatant above the polylysine-treated or control cells or the cell pellet was studied. Inorganic phosphate, carbohydrate, free amino acids, small peptides, potassium, and adenosine-5'-monophosphate were released from the tumor cells into the supernatant after polylysine treatment. Polylysine blocked the release of proteins and phospholipides, which passed into the suspending medium from the control cells. Antisera against Ehrlich ascites cells caused a similar change in morphology and leakage and retention of compounds from the tumor cells. This suggests that antibody-complement and polylysine may affect similar sites.

The basic polypeptide polylysine has been shown to inhibit growth and division of a diploid strain of Ehrlich ascites carcinoma and the TA3 ascites carcinoma in Swiss and BAF1 mice (13). These workers demonstrated that polylysine caused negligible change in cell volume. It has been observed that protamine, histone (3), and polylysine1 caused cytoplasmic aggregation and chromatin clumping in these cells. Morphological studies on the tumor cells treated with polylysine indicated that the cell membrane was not ruptured and that fluorescent polylysine did not penetrate the plasmalemma in sufficient quantities to be visible but rather was bound to the lipoprotein of the cell membrane.

In the present study the concentrations of various compounds released by the cells into the suspending medium after polylysine treatment were determined. The results showed that the polycation had a marked effect on the transport process of the tumor cell membrane.

MATERIALS AND METHODS

The cells used in this study were a diploid strain of Ehrlich ascites cells and were taken from Swiss mice 8 days following transplantation. Immediately after withdrawal, the cells were washed 3 times with a volume of saline equal to the amount of ascites fluid removed and then were suspended in this same volume of 0.9 per cent aqueous sodium chloride solution. There were about 1X10^6 cells/ml of suspension as determined from hemacytometer counting. To aliquots of this cell suspension was added a constant volume of either polylysine in saline or saline alone. The control cells are those diluted with saline but not treated with polylysine.

Polylysine was prepared by polymerization of ε-carbobenzyoxy-L-lysine anhydride with ammonia used as initiator. The molecular weight of the polylysine is a function of the ratio of anhydride to initiator during polymerization. The polylysine used in the following studies, except where otherwise indicated, was prepared with anhydride to initiator ratio of 3:0. The saline solutions of polylysine hydrochloride were adjusted to pH 7.0 with sodium bicarbonate before addition to the cells. The washed cells were suspended in different concentrations of polylysine for 10 minutes at room temperature. Then the cell suspension was centrifuged, and the supernatant or the cell pellet was used for the various analyses.

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The carbohydrate concentration in the saline media above the polylysine-treated and control cells was determined by the anthrone method (17); phosphate by the method of Fiske and Subbarow (17). The absorption of ultraviolet light by the supernatant was measured between 530 and 220 m\(\mu\) in a model 11 Cary recording spectrophotometer. Incorporation of \(\text{P}^{32}\) into the tumor cells \textit{in vitro} was estimated by the method of Creaser \textit{et al.} (6). In this method the phosphate incorporated into the cellular RNA, DNA, inorganic phosphate, and phospholipid is determined by extracting these components separately in different solvents. The free amino acid content of the supernatant above the polylysine-treated and control cells was measured by chromatography on an ion exchange resin (15) with the Beckman-Spinco automatic amino acid analyzer. The potassium and sodium concentrations of the polylysine-treated and control cells were determined by flame photometry with the Beckman DU flame spectrophotometer. The sodium content was determined by reading the optical density at 589 m\(\mu\), whereas potassium was measured at 768 m\(\mu\). The sodium content determined may be higher than was actually present in the cell because of the sodium chloride in the suspending medium. The proteins of the supernatants were studied by starch-gel electrophoresis in 0.25 m borate buffer at pH 8.6. Electrophoresis was run for 6 hours at 5.7 v/cm. Amido black was used for staining the protein bands. The lipide content of the ascites fluid above the unwashed cells with and without polylysine treatment was determined by the method of Freeman \textit{et al.} (7). The lipides were extracted from the ascites fluid with ethyl ether and then were chromatographed on a silicic acid-celite column. Three solvents were used to elute the three fractions: 5 per cent chloroform in hexane for cholesterol esters, chloroform for glycerides and free fatty acids, and methanol for phosphatides. These fractions were dried, then dissolved in carbon disulfide, and the infrared absorption spectrum was taken in the Baird Atomic infrared spectrophotometer. This determines quantitatively the lipide concentration of the three fractions.

RESULTS

Respiration studies indicated that the concentration of polylysine used in the present experiments caused less than 15 per cent inhibition of oxygen uptake by the tumor cells. Morphological studies of the tumor cells after polylysine treatment demonstrated that the plasmalemma was continuous, which indicated that cell lysis had not occurred.\(^1\)

RELEASE OF COMPOUNDS FROM TUMOR CELLS \textit{AFTER POLYLYSINE TREATMENT}

To 3 ml. of washed cells suspension was added 0.5 ml saline containing various concentrations of polylysine. After 10 minutes' incubation at room temperature the cell suspension was centrifuged, and the supernatant above the polylysine-treated and control cells was analyzed for carbohydrate, ultraviolet-absorbing compounds, phosphate, and amino acid content. The results are given below.

Carbohydrate.—One hundred \(\mu\)g. of polylysine/ml suspension caused the release of the equivalent of 10-12 \(\mu\)g glucose/ml. In one experiment the supernatant above the polylysine-treated cells had the equivalent of 40 \(\mu\)g glucose/ml, whereas that above the control cells had the equivalent of 30 \(\mu\)g glucose/ml. In another experiment the supernatant above the treated cells had the equivalent of 25 \(\mu\)g., whereas that above the control cells had the equivalent of 12 \(\mu\)g. glucose. Nirenburg (19) reported that Ehrlich ascites cells had a total polysaccharide content of 0.48 \(\mu\)moles glucose equivalents/gm protein.

Ultraviolet absorbing compound.—The absorption spectra of the supernatants above the treated and control cells revealed that polylysine caused the release of 260 \(\mu\)m absorbing material. The ultraviolet-absorbing compound present in greatest concentration moved the same as adenosine-5'-monophosphate, when paper chromatography with 3 per cent isopropanol, 19 per cent water, and 78 per cent saturated ammonium sulfate in water was used as solvent. The supernatant above the control cells had the equivalent of 10 \(\mu\)moles adenosine monophosphate/liter, as did the supernatant above the cells treated with 17.5 \(\mu\)g. polylysine. The supernatant above the cells treated with 175 \(\mu\)g. polylysine had the equivalent of 32 \(\mu\)moles/liter, whereas that above the cells treated with 1750 \(\mu\)g. polylysine had the equivalent of 26.7 \(\mu\)moles adenosine monophosphate/liter. This indicated that 175 \(\mu\)g. caused release of almost all the ultraviolet-absorbing material that is liberated by polylysine.

Phosphate.—The phosphate content of the supernatant above the polylysine-treated and control cells was determined. The supernatant above the cells that were not treated with polylysine contained 0.63 \(\mu\)moles phosphate/ml. In the supernatant above the cells that were treated with 17.5 \(\mu\)g. polylysine, there was 1.46 \(\mu\)moles phosphate/ml, whereas in the supernatant above those treated with 175 \(\mu\)g. polylysine there was 2.78 \(\mu\)moles phosphate/ml. Higher concentrations of polylysine
caused no increase in the phosphate released. Almost all the released phosphate was present as inorganic phosphate. There was a fourfold increase in the phosphate content of the supernatant after treatment of the cells with 175 µg, polylysine. Chance et al. (4) reported that Ehrlich ascites tumor cells contain 17 µmoles inorganic phosphate/gm wet weight.

To determine whether the phosphate metabolism of the cell was impaired by polylysine, P32 incorporation into the various fractions of the cell was determined with and without polylysine addition to the cell medium. This investigation was done in three parts. One test tube had no polylysine added, the second had polylysine added simultaneously with the P32, and the third had polylysine added 20 minutes after the P32 was added. Centrifugally packed Ehrlich ascites cells were diluted 8 times with the modified Krebs salt solution used by Creaser et al. (6). One ml. of this cell suspension was added to 3 ml. of this salt solution containing 100 µc. P32 or 100 µc. P32 and 400 µg. polylysine. The control and the experiment in which polylysine was added 20 minutes after P32 addition had only the P32 at zero time. For the latter experiment 0.3 ml. salt solution containing 400 µg. polylysine was added to the 3.7 ml. of cell suspension containing P32 and salt solution after 20 minutes. Three-ml. aliquots were removed from each of the test tubes at 15- and 30-minute time periods after the addition of P32. The cells were then washed, the various cellular compounds extracted, and 0.4 ml. of each extraction fraction was put on a planchet and counted. The results are presented in Table 1. The P32 incorporated above the control cells and those treated with 400 µg. polylysine/ml were analyzed for the concentration of free and bound amino acids. Free amino acids were determined after picrate deproteinization according to the method of Stein and Moore (16). The analyses were made on the Spinco amino acid analyzer at 50°C on the 150-cm. column (15). The eluting solvent was 0.2 N Na citrate at pH 3.25 and 4.25. The results of these experiments are seen in Table 2. When the free amino acids above the tumor cells treated with 400 µg polylysine/ml were analyzed, five unidentified acidic peaks were observed, and a sixth peak after serine was identified as glutathione by its elution position. The five peaks appeared at 25, 32, 62, 78, and 90 ml. of effluent in the above system. Upon hydrolysis of this picric acid-soluble fraction with 6 N HCl these six peaks were destroyed, and there was a twofold increase in the aspartic and glutamic acid peaks. The increase in these simultaneou

### Table 1

<table>
<thead>
<tr>
<th>Extract</th>
<th>No polylysine</th>
<th>Polylysine at zero time</th>
<th>Polylysine at 10 min.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>15 min.</td>
<td>30 min.</td>
<td>15 min.</td>
</tr>
<tr>
<td>Acid-soluble fraction:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. No. 1</td>
<td>600</td>
<td>940</td>
<td>240</td>
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<tr>
<td>&quot; &quot; 2</td>
<td>617</td>
<td>940</td>
<td>250</td>
</tr>
<tr>
<td>Phospholipide:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. No. 1</td>
<td>6.12</td>
<td>10.8</td>
<td>4.63</td>
</tr>
<tr>
<td>&quot; &quot; 2</td>
<td>6.62</td>
<td>11.1</td>
<td>5.18</td>
</tr>
</tbody>
</table>

* Results are expressed as counts × 10^3 per minute incorporated in cells per 3-ml. aliquot.
two amino acids with no comparable release of other neutral or basic amino acids suggests that the five acidic peaks in the free amino acids fraction may be peptides of the acidic amino acids or amino acid-nucleic acid conjugates. Agren has recently reported (1) on the occurrence of acid-soluble nucleotide-linked peptides in trichloroacetic acid extracts of Ehrlich ascites cells. When these nucleotide-linked peptides were applied on glutamic acid, and aspartic acid were released. If the amino acid analyzer it might be expected that acid hydrolysis of these peaks glyeine, serine, mate, six peaks were separated which came off the column where adenosine-diphosphate did. Upon ~ml. aliquot of supernatant above polylysine-treated and con-

acetic acid extracts of Ehrlich ascites cells. When these peaks were chromatographed on Dowex 1 and eluted with formic acid and ammonium formate, six peaks were separated which came off the column where adenosine-diphosphate did. Upon acid hydrolysis of these peaks glycine, serine, glutamic acid, and aspartic acid were released. If these nucleotide-linked peptides were applied on the amino acid analyzer it might be expected that they would be eluted very rapidly. Some of our unidentified peaks may well be the nucleotide peptides.

From Table 2 it is clear that polylysine caused a large release of free taurine. The total free amino acid content of the Ehrlich ascites cells was not determined by the authors; however, Sassenrath et al. (14) have reported the content of aspartic, glutamic, glycine, and alanine in these cells. Two-ml. cell volume contains 0.61 μmoles aspartic acid, 3.30 μmoles glutamic acid, 5.20 μmoles glycine, and 5.8 μmoles alanine in the free form.

Potassium and sodium gradient of the cell.—To 2 ml. of tumor cells in ascites fluid was added either 1 ml. of 0.9 per cent sodium chloride solution or 1 ml. containing 800 μg polylysine dissolved in saline. The polylysine was prepared with an anhydride to initiator ratio of 170. The mixture was incubated for 15 minutes at 28°C, and then the cells were centrifuged and washed twice with saline. The packed cells were dried for 90 minutes at 100°C. Then 0.16 gm. of cells were taken from each sample and heated for 1 hour at 150°C, then at 270°C for 1 hour, and finally at 500°C for 3 hours. The ash was dissolved in 0.4 N HCl. A standard curve for sodium and potassium was determined for the flame photometer. The effect of polylysine on the cellular content of these two monovalent cations is presented in Table 3 The cellular potassium concentration of the control cells is similar to that reported by Christensen, who found 108 meq. K+ per liter (5). From this it can be seen that the potassium and sodium gradient of the cell has been destroyed by polylysine.

Retention of proteins by tumor cells after polylysine treatment.—Ten-ml. aliquots of washed tumor cells suspended in saline were treated with two concentrations of polylysine (50 and 500 μg polylysine/ml). The cells were incubated at room temperature for 15 minutes, and then the cells were centrifuged and 0.05 ml. of the supernatant was analyzed for free amino acids or total amino acids (free+peptide+protein) by ion exchange chromatography.

The potassium and sodium gradient of the cell has been destroyed by polylysine.
proteins in the supernatant to the cell surface or prevent the release of cellular proteins into the suspending medium. Dr. L. Hokin found that protamine sulfate (a polycation similar to polylsine) inhibited the release of amylase from the carbamyl cholinestimulated pancreas (personal communication). This inhibition may be due to protamine binding to the surface of the pancreatic duct, thereby preventing protein secretion.

Retention of phospholipides by the tumor cell after protamine treatment.—Two ml of washed tumor cells suspended in saline were treated with 200 μg polylsine/ml for 15 minutes at room temperature. The cells were then centrifuged, and the lipide soluble fraction of the supernatant treated as described in “Materials and Methods.” There was no difference in the cholesterol esters or in the neutral fat content in the supernatant above the polylysine-treated or untreated cells. However, there was a marked decrease in the infrared absorption at 1100 cm⁻¹ of the supernatant above the polylysine-treated cells as compared with the untreated cells. The infrared absorption curves of the ether extracts from the supernatants above the polylysine-treated and control cells were compared. The absolute concentration of the lipides absorbed at this wave number, this decrease suggested that polylysine prevented release of phospholipides from the tumor cells. The negative charge on the phospholipides may be a binding site for the polylsine.

DISCUSSION

It has been shown in this study that polylysine impaired the capacity of the Ehrlich ascites tumor cell membrane to retain small molecular weight materials such as nucleotides, amino acids, peptides, potassium, carbohydrate, and phosphate. In contrast, polylysine prevented the loss of proteins and phospholipides which were released from the untreated control cells. Green et al. reported that complement, together with antibodies prepared against Ehrlich ascites cells, caused the release of amino acids, phosphate, monosaccharides, and less leakage of proteins and phospholipides from the Ehrlich ascites cell (10). Ginsburg reported (9) that phospholipides and proteolipides prevent the cytotoxic effect of anti-Ehrlich ascites serum by combining with complement. Becker (8) has recently demonstrated that phospholipides inhibit the hemolysis of red blood cells caused by the polycation protamine.

The release of small molecules and retention of large molecules by tumor cells exposed to polylysine or to anti-ascites sera, and the similar changes in morphology induced by both (11), suggest that the binding site of antibody-complement and polylysine to the cell may be similar. This is supported by the capacity of phospholipides to inhibit these changes induced by both polycation and antibody.

The release of potassium from the tumor cells may cause the observed cytoplasmic aggregation and the chromatin clumping. The binding of phospholipide by the polycation may produce an inhibition of protein synthesis as reported by Green (10), since phospholipide is involved in intracellular transport across the cell membrane (8). Polylsine inhibited the incorporation of P³² into the phospholipide fraction of the tumor cells. This may be the result of polycation—phospholipide interaction. The release of adenine-5'-monophosphate from the tumor cell after polylysine treatment may reduce protein synthesis. The disruption of the potassium gradient in the cell will probably affect the metabolism of the cell as well.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1.—Effect of polylysine on the release of protein by the Ehrlich ascites tumor cell. Tumor cells were incubated with different concentrations of polylysine for 15 minutes, and then a 0.05-ml aliquot of supernatant above these cells was subjected to electrophoresis. Pattern A is the supernatant above the untreated cells, B is that above the cells treated with 50 μg polylysine/ml, C is that above the cells treated with 500 μg polylysine/ml.
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