The Role of Sialic Acid in the Release of Proteins from L1210 Leukemia Cells

J. LESLIE GLICK,2 ALLAN R. GOLDBERG, AND ARTHUR B. PARDEE

Department of Biology, Princeton University, Princeton, New Jersey

Summary

The presence of sialic acid on cell membranes was found to be necessary for the outward flow of proteins from L1210 leukemia cells. The removal of membrane-bound sialic acid inhibited the release of proteins without any effect on cell lysis, or on the release of nucleotides or sugars. The requirement for sialic acid in promoting protein secretion seemed relatively specific for certain proteins, as determined by means of disc gel electrophoresis.

Introduction

In recent years several laboratories have reported that ascites tumor cells release large quantities of enzymes into the surrounding medium in vitro (1, 6, 9, 18). The interstitial fluid of tumors has also been found to contain a variety of proteolytic enzymes (13, 14). The nature of the mechanisms controlling the release of these enzymes has not been explored.

Preliminary studies have shown that sialic acid, which is present in great amounts on the cell membranes of various tumor strains (2, 4, 8, 15, 16), modifies potassium transport in L1210 leukemia cells (5). The removal of membrane-bound sialic acid inhibited potassium flow either inward or outward, depending on the incubation conditions. However, sodium, glucose, and lysine fluxes were unaffected. In the present study we have found that the removal of sialic acid also impedes the release of certain proteins from the L1210 cells.

Materials and Methods

L1210 leukemia cells (donated by Dr. D. J. Hutchison, Sloan Kettering Institute, Rye, New York) were maintained in the ascitic form in male B6D2Fi mice weighing 20-25 gm (supplied by Jackson Memorial Laboratory, Bar Harbor, Maine). Experiments were conducted with L1210 cells that had been isolated, centrifuged, and washed at 3°C with isotonic saline until free of red blood cells. The L1210 cells were suspended in 25-ml flasks, containing 9 parts of isotonic saline and 1 part of 0.10 M sodium phosphate buffer (pH 7.0). Glass beads were added to each flask in order to prevent the cells from clumping. The cells were incubated at 37°C for 90 min in a slowly moving New Brunswick gyratory shaker (New Brunswick, New Jersey). They were then centrifuged, and the supernatant was collected for chemical determination.

During certain incubations sialic acid was removed from the L1210 cells by the addition of 250-500 units of Vibrio cholerae neuraminidase (obtained from General Biochemicals, Chagrin Falls, Ohio). Under these conditions the sialic acid released was derived essentially from the cell membrane (15). Free sialic acid was determined by the thiobarbituric acid assay of Warren (17) and also by the resorcinol method of Svennerholm (11). Both of these analyses were performed with either a Zeiss spectrophotometer or a Beckman spectrophotometer.

The amounts of protein released by the cells into the incubation media were assayed by the phenol reagent procedure of Lowry et al. (7). Canalco disc gel electrophoresis equipment (Bethesda, Maryland) was used to separate the proteins in a 0.05 M tris(hydroxymethyl)aminomethane-0.38 M glycine buffer (pH 8.3). The protein bands were then stained with naphthol blue black (0.55 gm/100 ml of 7.5% acetic acid), and the band densities were recorded with a Joyce microdensitometer (Gateshead, England).

Results and Discussion

As determined by the thiobarbituric acid assay, 0.30 µmole of sialic acid/10⁶ cells was removed by neuraminidase. No sialic acid was found in the supernatant of control cells.

When the sialic acid content of the experimental supernatant (obtained from neuraminidase-treated cell suspensions) was measured by the resorcinol method, some unexpected results were obtained. This technic normally results in absorption maxima at 580 and 450 mµ due to pure sialic acid; the absorbancy coefficients are 9400 and 3100 liters/mole, respectively, and the absorbancy ratio (Asm/Aw) is 3.03. As shown in Table 1, the absorbancy ratio of the experimental supernatant was 81% higher than that of the control supernatant, thereby indicating that the neuraminidase-treated cells released sialic acid. However, the experimental absorbancy ratio of 1.83 was appreciably lower than that for pure sialic acid. This result suggested that the neuraminidase-treated cells released some unidentified substance(s), in addition to sialic acid, which reacted with the resorcinol reagent. Furthermore, the absorbancy of the experimental sample at 450 mµ was 32% lower than that of the control. We propose that the control cells also released some unidentified substance(s).

---

Received for publication November 12, 1965; revised March 21, 1966.

1 Supported by Grants 1-F2-CA-24245-01, 5T1-GM-457, and A1-04409 from the USPHS.
2 Present address: Cell Laboratories, Roswell Park Memorial Institute, Buffalo, New York.

1774
TABLE 1
INHIBITION OF THE RELEASE OF UNIDENTIFIED SUBSTANCE(S) FROM L1210 CELLS UPON THE REMOVAL OF SIALIC ACID

The control group represents those supernatants which were obtained from suspensions of cells not treated with neuraminidase. The experimental group represents those supernatants from cells which were treated with neuraminidase. The supernatants from both groups were processed by the resorcinol procedure. The absorbancies of the supernatants were measured at 450 and 580 μm. Each value reported is the mean of 7 experiments, ± the S.E. Percentage changes from the control values are given in parentheses. Statistical significance was determined by means of the "t" test for paired variates. See text for interpretation of the data.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>ABSORBANCY UNITS/10⁹ CELLS</th>
<th>ABSORBANCY RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A₄₅₀¹</td>
<td>A₅₈₀ᵇ</td>
</tr>
<tr>
<td>Control</td>
<td>3.16 ± 0.80</td>
<td>3.03 ± 0.70</td>
</tr>
<tr>
<td>Experimental</td>
<td>2.14 ± 0.78 (−32)ᵇ</td>
<td>2.87 ± 0.66 (−5)ᵇ</td>
</tr>
</tbody>
</table>

* Change in absorbancy at 450 μm from 0 to 90 min.
ᵇ Change in absorbancy at 580 μm from 0 to 90 min.
ᵇ P < 0.01.
bᵇ P < 0.05.

TABLE 2
THE EFFECT OF NEURAMINIDASE ON A SUPERNATANT FROM A CONTROL CELL SUSPENSION

The supernatant was obtained after the cells were incubated for 90 min in the absence of neuraminidase. The supernatant was then divided between 2 tubes; neuraminidase was added to 1 of the tubes, and both portions were reincubated from 90 to 180 min. The colorimetric assay was performed according to the resorcinol procedure. Each value represents the mean of duplicate determinations. Percentage changes from the control values are given in parentheses.

<table>
<thead>
<tr>
<th>ADDITION</th>
<th>ABSORBANCY UNITS/10⁹ CELLS</th>
<th>ABSORBANCY RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A₄₅₀¹</td>
<td>A₅₈₀ᵇ</td>
</tr>
<tr>
<td>None</td>
<td>2.86</td>
<td>3.07</td>
</tr>
<tr>
<td>+ Neuraminidase</td>
<td>3.04 (+6)</td>
<td>3.10 (+1)</td>
</tr>
</tbody>
</table>

* Change in absorbancy at 450 μm from 0 to 180 min.
ᵇ Change in absorbancy at 580 μm from 0 to 180 min.

substance(s), and that the removal of sialic acid apparently inhibited the release of the unidentified substance(s). The similarity of the absorbancies obtained from the control and experimental samples at 580 μm was probably coincidental. Thus, at this particular wave length the control absorbancy, which was caused solely by the presence of the unidentified substance(s), equalled the experimental absorbancy, which was due to both the accumulation of sialic acid and the partial disappearance of the unidentified substance(s).

The experiment reported in Table 2 was designed to test if neuraminidase, per se, was able to affect the absorbancy of the supernatant at 450 and 580 μm by reacting directly with the unidentified substance(s) released into the medium. L1210 cells were incubated for 90 min without neuraminidase. The cells were then centrifuged, and the supernatant was divided between 2 test tubes. One of the test tubes contained neuraminidase, and the other contained saline. The supernatant was incubated for an additional 90 min and then was prepared according to the resorcinol procedure. The changes in absorbancy at 450 and 580 μm over the total 180-min period did not differ between the control supernatant and the enzyme-treated supernatant. This experiment reinforced the results summarized in Table 1, by demonstrating that the inhibition in the release of any unidentified substances from cells stripped of sialic acid was not an artifact due to the action of neuraminidase on these substances.

By means of the resorcinol procedure, a method was next devised to estimate both the amount of sialic acid removed by neuraminidase and the corresponding degree of inhibition in the release of the unidentified substance(s). Simultaneous equations were set up for the absorbancies at 450 and 580 μm in terms of the concentrations of sialic acid and the unidentified substance(s), and their absorbancy coefficients.

TABLE 3
INHIBITION OF THE RELEASE OF UNIDENTIFIED SUBSTANCE(S) FROM CELLS STRIPPED OF SIALIC ACID: AGREEMENT BETWEEN 2 DIFFERENT DETERMINATIONS

Each of the values determined by means of Calculation I is the mean of 7 experiments, ± the S.E. Each of the values determined by means of Calculation II is the mean of 3 experiments, ± the S.E.

<table>
<thead>
<tr>
<th>Calculations</th>
<th>Removal of sialic acid (μmole/10⁹ cells)</th>
<th>% inhibition in release of unidentified substance(s)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iᵃ</td>
<td>0.29 ± 0.05</td>
<td>41 ± 9</td>
</tr>
<tr>
<td>IIᵇ</td>
<td>0.30 ± 0.02</td>
<td>43 ± 9</td>
</tr>
</tbody>
</table>

ᵃ Sialic acid removed by neuraminidase during a 90-min incubation.
bᵇ Percentage decrease from the control value, in the absence of neuraminidase.
ᵇ Calculation I was applied to the results obtained from the resorcinol procedure, as explained in the text.
ᵇ Calculation II was applied to the results obtained from both the thiobarbituric acid assay and the resorcinol procedure, as explained in the text.

AUGUST 1966 1775
Here, \( A_1 \) and \( A_2 \) are absorbancies at 450 and 580 m\( \mu \), respectively. \( C_s \) is the concentration of sialic acid, and \( C_{un} \) is the concentration of the \( n \)th unidentified substance. \( E_i \)'s are absorbancy coefficients of the compounds at wavelengths indicated by subscripts.

A simplifying assumption must now be made, since neither the concentrations nor the absorbancy coefficients of the unidentified compounds are known. It will be assumed that the type(s) of unidentified substance(s) released into the medium will be the same in the control and the experimental samples. The total concentration of the unidentified mixture will be defined as \( C_{un} \), and its average absorbancy coefficients will be defined as \( E_{iu} \) and \( E_{iu} \) at the two wave lengths. (This is equivalent to considering the unidentified mixture as a single entity.) The above equations may then be reduced to the following form:

\[
\begin{align*}
A_1 &= C_s E_{iu} + \sum_n C_{un} E_{iun} \\
A_2 &= C_s E_{iu} + \sum_n C_{un} E_{iun}
\end{align*}
\]

Finally, since no sialic acid is present in the control supernatant, \( C_s = 0 \), and \( A_1/A_2 = E_{iu}/E_{iu} = 1.0 \) (Table 1). We now have 2 equations which permit us to calculate \( C_{un} \), the sialic acid content in the supernatant of the experimental sample. We can also compute the ratio of the unidentified material in the 2 samples and thus determine the percentage inhibition in the release of unidentified substance(s) from the neuraminidase-treated cells. These results are presented in Table 3.

The calculation obtained by means of the resorcinol procedure for the amount of sialic acid removed by neuraminidase agrees closely with the value obtained by the more direct thiobarbituric acid assay. If the contribution of this latter value is subtracted from the experimental absorbancy found according to the resorcinol method, the remaining absorbancy should be attributed to the unidentified substance(s). As shown in Table 3, the percentage inhibition in the release of unidentified material from cells stripped of sialic acid was approximately 40%, regardless whether the resorcinol procedure was utilized solely or in conjunction with the thiobarbituric acid assay.

In order to identify the substance(s) whose release was inhibited from the neuraminidase-treated cells, we employed thin layer chromatographic and ultraviolet spectrophotometric techniques for detecting nonamino sugars and nucleotides. The removal of sialic acid from the membranes apparently had no effect upon the release of these metabolites.

We then studied the release of proteins from the L1210 cells.
and indeed found that protein release was inhibited from cells stripped of sialic acid. The percentage inhibition was 40 ± 4%, and was almost identical with that calculated for the unidentified substance(s), (Table 3). Table 4 demonstrates that the greater amount of protein released from the control cells could not have been due to increased lysis during the 90-min incubation period. Cell counts showed that about 30% of the cells were lysed by 90 min in both the control and experimental groups. Cell viability, as determined by eosin staining (3), was not affected by the action of neuraminidase. The amount of protein accounted for by cell lysis was therefore the same in both groups. The net protein released, i.e., protein not due to cell lysis, was about 3 times greater from the control cells than from the neuraminidase-treated cells.

Electrophoretic patterns were obtained from the cellular proteins released into the incubation medium. Nine major protein bands were observed in each gel. There were no discernible differences in the number and location of the protein bands, when one compared the experimental samples to the control samples. However, as shown in Table 5 (which depicts a typical experiment), the densities of 3 bands (a, b, and f) were depressed upon the removal of sialic acid. Five other bands were not affected, and the density of 1 other band (c) was actually enhanced by the loss of sialic acid. When the data from 3 of these experiments were pooled, the differences between the control and the experimental groups were statistically significant only with respect to bands a, b, c, and f. These differences are depicted most graphically in Chart 1.

The above electrophoretic results raise several points worthy of further study. The release of proteins was selectively affected by removing sialic acid from the L1210 cells. A number of protein bands were not affected at all, and presumably these bands represent at least some of the proteins released during cell lysis. Those band densities which were depressed in the experimental samples were not inhibited equally. The percentage of inhibition in the density of band a was 2 times greater than that of bands b or f. Moreover, the unique stimulation of the density of band c in the experimental sample offers a possible explanation for the control of protein secretion in the L1210 cell. The presence of the negatively charged sialic acid on the cell membrane might be required in order to bind a specific protein which is necessary for transporting intracellular proteins out of the cell. The enzymatic removal of the membrane-bound sialic acid would cause the release of the "transport protein," which in turn would block the secretion of intracellular proteins. Such a mechanism, involving the binding of a "transport protein" to membrane-bound sialic acid, might be advantageous for a tumor cell when characteristically releasing large quantities of intracellular enzymes (1, 6, 9, 13, 14, 18). The production and leakage of these enzymes have been related to the invasiveness of tumor cells and the resulting damage inflicted upon the normal host tissue (12). Thus, alterations in the surface properties of tumor cells may contribute to the unchecked growth of the tumor (10).

Acknowledgment

The technical assistance of Miss E. Robinson is gratefully acknowledged.

References

The Role of Sialic Acid in the Release of Proteins from L1210 Leukemia Cells

J. Leslie Glick, Allan R. Goldberg and Arthur B. Pardee

*Cancer Res* 1966;26:1774-1777.

| Updated version | Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/26/8_Part_1/1774 |

| **E-mail alerts** | Sign up to receive free email-alerts related to this article or journal. |
| **Reprints and Subscriptions** | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| **Permissions** | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |