Effect of Lysolecithin and Analogs on Mouse Ascites Tumors

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ABSTRACT

Antitumor activity of lysolecithin and its ester and ether analogs has been investigated in three mouse ascites tumors: Meth A, Ehrlich, and Sarcoma S180J. Immediately after mice were inoculated i.p. with a suspension of tumor cells, they were given a single i.p. injection of lysolecithin or an analog. Inhibition of tumor growth was measured as the total packed cell volume on Day 8.

Lysolecithin induced tumor inhibition against Ehrlich and S180J and, to a lesser extent, against Meth A ascites tumor. Further tests with ester-deoxylysolecithin analogs against Meth A showed that derivatives with stearic or lauric acid esters on the C-1 atom of the propanediol moiety induced tumor inhibition to a variable degree. However, the ether analogs, 1-hexadecyl or 1-dodecylpropanediol-3-phosphorylcholines and α,1,2-dioctylglycerol-3-phosphorylcholine, but not the 1-decylpropanediol-3-phosphorylcholine, produced more marked, reproducible inhibition.

Detergents, sodium dodecyl sulfate and Hyamine 10-X hydroxide, when injected i.p. immediately after ascites cells, reduced the capacity of the tumor cells to multiply as indicated by lower total packed cell volume on Day 8. Tween 80 did not produce this effect at the same dose. The effect of lysolecithin against the Meth A ascites was observed when treatment was administered 4 days before tumor inoculation but not before that day or subsequently. 1-Hexadecylpropanediol-3-phosphorylcholine was effective when administered as early as 7 days before tumor inoculation or as late as 2 days after.

Treatment with lysolecithin of mice inoculated with 0.5 to 2 x 10⁶ Meth A cells significantly prolonged the median survival time of tumor hosts.

Exposure of ascites cells of Meth A tumor to lysolecithin, 1-hexadecylpropanediol-3-phosphorylcholine, or Hyamine 10-X hydroxide destroyed the cells as shown by trypan blue staining and by reduction of the capacity of the cells to produce tumors on intradermal injection into mice. The concentrations of these substances required to produce a 50% effect were similar over a narrow range. Far higher concentrations of sodium dodecyl sulfate were required for a comparable effect.

INTRODUCTION

Among chemicals and biological products that damage the cell membrane of tumor cells, while affecting normal cells to a smaller degree or not at all, lysolecithin has attracted the attention of several investigators. Fischer (5) observed that small concentrations of lysolecithin reduced respiration of Ehrlich ascites tumor cells, while the oxygen consumption of suspensions of micro- and macrophages was increased. Mehrishi and Butterworth (9) have shown that lysolecithin causes release of potassium from BP8 ascites tumor cells and normal murine lymphocytes and that the effective concentration of lysolecithin in terms of cell surface area was smaller in BP8 than in normal murine lymphocytes. Human leukemic cells were lysed by lower concentrations of lysolecithin than were normal human leukocytes (7).

When synthetic analogs of lysolecithin became available (2-4, 16), Langer (8) and Munder et al. (12) examined the effect of lysolecithin and of its analogs on the growth of Ehrlich and Meth A mouse fibrosarcoma ascites tumors and found that the ability to suppress the growth of tumors depended on the chain length of the substituent fatty acid on C-1 of the glycerol or propanediol moiety of the lysolecithin analogs. The effect appeared to be caused by stimulation of cell-mediated and humoral immune effector mechanisms of the host.

This paper describes the results of our investigation of the effects of lysolecithin and its analogs on several ascites tumors; this study is a part of a larger program seeking immunomodulating substances that are endowed with antitumor activity (15). In an attempt to examine the part played by the lysis of tumor cells caused by the detergent activity of lysophospholipids in the antitumor effects of these chemicals, the in vitro and the in vivo activities of 3 synthetic detergents have been investigated.

MATERIALS AND METHODS

Ascites forms of Meth A fibrosarcoma, Ehrlich tumor, and S180J of the mouse were used in this study. Meth A was maintained in BALB/c mice from the Sloan-Kettering Institute mouse colony and tested in male BALB/cJ x C57BL/6J F₁ (hereafter called CB6F₁) mice from the Jackson Laboratory, Bar Harbor, Maine. S180J was maintained and tested in ICR/Ha-derived female CD-1 mice from the Charles River Mouse Farms, Inc., Wilmington, Mass., and Ehrlich ascites tumor in Swiss-Webster female mice from Taconic Farms, Inc., Germantown, N. Y. Mice weighing 18 to 22 g at the time of tumor implantation were given water and food ad libitum.

Lysolecithin was purchased from General Biochemicals, 82001, U.S.A.
Inc., Chagrin Falls, Ohio, and lysolecithin analogs were synthesized by H. U. Weltzien, O. Westphal, D. Arnold, and H. Eible (2-4, 16). Lysolecithin, immediately upon receipt, was dissolved in absolute ethanol and stored at 10°C before administration, lysolecithin solution was diluted with pyrogen-free 0.85% NaCl solution. Lysolecithin analogs were dissolved directly in pyrogen-free 0.01 M phosphate-0.85% NaCl solution. Sodium dodecyl sulfate was purchased from Matheson Coleman and Bell, Norwood, Ohio; Hyamine 10-X hydroxide [p-(diisobutylcresoxyethoxyethyl)-dimethylbenzylammonium hydroxide] solution in methanol was from the Packard Instrument Co., Downers Grove, Ill., and Tween 80 (polyoxyethylene sorbitan monooleate) was from Schwarz/Mann, New York, N. Y.

Washed ascites tumor cells suspended in Earle’s balanced salt solution were counted as viable cells by trypan blue exclusion. One-ml aliquots containing 1 x 10⁶ viable tumor cells were injected i.p. on Day 0 of the experiment. The TPCV of ascites tumors was determined on Day 8, as previously described (13). In other experiments, the median survival time of the hosts of ascites tumors was determined.

The in vitro cytotoxic effect of lysophospholipids and synthetic detergents was examined with the use of suspensions of washed Meth A ascites cells in Earle’s balanced salt solution containing 1 x 10⁶ tumor cells/ml and graded concentrations of test chemicals. After 1-hr exposure to chemicals at room temperature in air, the incubation medium was removed by centrifugation and cell pellets were resuspended in Earle’s balanced salt solution free of test chemical. The percentage of tumor cells staining with trypan blue was then determined, and parallel aliquots of cell suspensions diluted to contain 1 x 10⁶ tumor cells in 0.2 ml volume were inoculated i.d. into male CB6F, mice. The number of mice in which solid tumors failed to develop at the end of 2 weeks was recorded.

In a series of 4 tests, groups of 5 male CB6F, mice were inoculated with suspensions containing increasing numbers of untreated Meth A cells. Inoculum ranged (at 2-fold increments) from 1 to 256 x 10⁶ cells/mouse i.d. The 50% end point, i.e., the size of tumor inoculum producing tumors in 50% of mice at 14 days after tumor inoculation, was 1.66 x 10⁴ cells/mouse.

The inocula of Meth A cells exposed to test chemicals in concentrations that had caused the tumor inocula not to produce tumors in 50% of inoculated mice thus contained at least 1.66 x 10⁴ viable Meth A cells.

RESULTS

The effect of lysolecithin on the ascites form of Ehrlich, Meth A, and S180J was assayed with doses ranging from 0.1 to 1.0 mg/mouse i.p. on Day 0, the day of implantation of ascites tumor, using inocula of 1 x 10⁶ cells i.p. Corresponding control mice were given injections of either 10% ethanol in 0.85% NaCl solution in the tests of lysolecithin or phosphate-buffered NaCl solution in tests of other chemicals. The effect was measured as the TPCV of ascites tumors on Day 8. Lysolecithin in doses from 0.25 to 1.0 mg/mouse prevented growth of EA and S180J. With Meth A, a variable response to lysolecithin was observed. In 3 tests (of 5), a dose of 0.5 mg/mouse (in 2 other tests, a dose of 1.0 mg/mouse) reduced the TPCV significantly. Lower doses of lysolecithin induced some reduction of all 3 tumors without completely preventing growth (Chart 1).

No noticeable toxic effect (compared to 0.85% NaCl solution control) on the body weight was observed in groups of 6 tumor-free male CB6F, mice, each given injections of lysolecithin, 1-stearoylpropanediol-3-phosphorylcholine, or 1-hexadecylpropanediol-3-phosphorylcholine, 1 mg/mouse i.p. No deaths due to toxicity were observed.

The ester-deoxy analogs of lysolecithin, 1-stearoyl- and 1-lauroylpropanediol-3-phosphorylcholines, produced in Meth A a variable response similar to that of natural lysolecithin. Of the 7 other analogs of lysolecithin tested against Meth A ascites tumor, 1-hexadecylpropanediol-3-phosphorylcholine (Chart 2) produced a consistent dose-related effect with almost complete prevention of tumor growth at the 2 highest doses. The analog 1-dodecylpropanediol-3-phosphorylcholine produced an antitumor effect similar to that of lysolecithin and the 2 ester-deoxy analogs, while the analog 1-decyloxylpropanediol-3-phosphorylcholine was completely inactive. The activity of the analog, 1,1-diocetylglycerol-3-phosphorylcholine carrying the C₂₀ alcohol substituents on both the C-1 and C-2 atoms of the glycerol moiety is comparable to that of 1-dodecylpropanediol-3-phosphorylcholine.

The C₁₈ ether analog substituted with a methyl group on the C-2 atom of the glycerol moiety was as active as 1-hexadecylpropanediol-3-phosphorylcholine, while, of the 2 analogs substituted with benzyl groups on the C-2 atom, the analog with the C₁₈ alcohol substituent on C-1 of the glycerol was active, whereas the analog with C₁₈ substituent was not at the tested dose (1 mg/mouse) (Table 1).

Of the 3 synthetic detergents tested in vivo, Tween 80 was ineffective in all doses, sodium dodecyl sulfate produced highly significant inhibition of tumor growth in doses of 0.5 and 1 mg/mouse, and Hyamine 10-X hydroxide inhibited tumor growth significantly at 0.25 and 0.5 mg/ml.

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* According to the information provided by the source, lysolecithin was prepared from egg lecithin by hydrolysis with snake venom; its fatty acids (predominantly palmitic and stearic acids in the ratio of 6:4) were primarily on C-1 of glycerol. The purity of lysolecithin was established by chromatography on silicic acid (V. P. Skipski, Memorial Sloan-Kettering Cancer Center, personal communication).

**Table 1**

<table>
<thead>
<tr>
<th>Analog substituted on</th>
<th>Overall TPCV (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>C-1</td>
<td></td>
</tr>
<tr>
<td>O-CH₃</td>
<td>1.24 ± 0.08³</td>
</tr>
<tr>
<td>O-CH₂CH₂</td>
<td>0.87 ± 0.19³</td>
</tr>
<tr>
<td>O-CH₂CH₃</td>
<td>1.06 ± 0.32³</td>
</tr>
<tr>
<td>C₂-ET</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.25³</td>
</tr>
</tbody>
</table>

³ 16-ET, hexadecyl ether; 18-ET, octadecyl ether.

* Mean ± S.D. (among triplicate experiments).

**Table 2**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Diluent mg/mouse</th>
<th>0.10</th>
<th>0.25</th>
<th>0.50</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>1.09 ± 0.21³</td>
<td>1.02 ± 0.16³</td>
<td>0.80 ± 0.17³</td>
<td>0.38 ± 0.06³</td>
<td>0.26 ± 0.10³</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1.09 ± 0.21³</td>
<td>0.98 ± 0.34³</td>
<td>1.28 ± 0.44³</td>
<td>1.26 ± 0.18³</td>
<td>1.07 ± 0.39³</td>
</tr>
<tr>
<td>Hyamine 10-X hydroxide</td>
<td>1.09 ± 0.21³</td>
<td>0.63 ± 0.21³</td>
<td>0.19 ± 0.19³</td>
<td>0.29 ± 0.19³</td>
<td>0.26 ± 0.10³</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.

* As in Table 1, Footnote c (p < 0.01).

and was lethal at 1 mg/ml (Table 2).

After we observed that lysolecithin and some of its analogs, when injected i.p. immediately after i.p. inoculation of Meth A ascites cells, were effective in reducing the volume of tumor cells that developed by 8 days, we explored further the time relationship between chemical and tumor inoculation compatible with effective reduction of tumor cell replication. To this end, groups of similar mice given Meth A ascites cells on Day 0 were treated with a single dose of lysolecithin or 1-hexadecylpropanediol-3-phosphorylcholine analog, 1 mg/mouse i.p., on Days −7, −4, −1, 0, 1, 2, or 4. Chart 3 shows the consistency of TPCV values in diluent controls at the various points of time, as well as the efficacy, with some irregularity of lysolecithin, in reducing tumor cell volume when administered on Day −4 or on Day 0, but not before or after. The effect of the analog was more striking and consistent, showing significantly lower TPCV when given on Days −7, −4, −1, 0, or 2 (p < 0.05), although not on Day 4.

The observed antitumor activity of lysolecithin, given before or on the same day as the Meth A cells, prompted the inquiry as to whether an enhanced effect could be achieved by combined pretreatment and simultaneous treatment. Two groups of mice were given lysolecithin in graded doses on Day 0; one-half of the mice had already been pretreated on Day −4 with lysolecithin, 1 mg/mouse, while the corresponding controls had been given diluent only. The effect of pretreatment on Day −4 is represented in Chart 4 by the difference between the height of the horizontal lines showing the value of TPCV at various doses of lysolecithin. The effect of pretreatment was demonstrable over the range of the lower doses from 0 to 0.25 mg/
TPCV (treated/simultaneous control). Asterisk, T/C was significantly lower given i.p. injections of lysolecithin, 1 mg/mouse; the other half were given TPCV, thus masking the enhancing effect of pretreatment.

Only, Days -4 and 0). Asterisk, T/C was significantly smaller than cumulated horizontal lines, with (•) and without (O) pretreatment with lysolecithin. T/C were given i.p. injections of graded doses of lysolecithin from 0 to 1 mg/mouse (p < 0.05). At the higher doses of 0.5 and 1 mg/mouse (a), or its analog, l-hexadecylpropanediol-3-phosphorylcholine. Each point represents the mean TPCV of 3 tests, each based on 5 treated or 5 control mice. T/C, the ratio of TPCV (diluent- or lysolecithin-pretreated on Day -4) to (diluent control-activity relationships of these compounds, some features of the dose-response curves obtained in the course of investigation of their effects on the TPCV of Meth A ascites merit comment. Meth A required higher concentrations of lysolecithin for inhibition, which was less consistent from test to test than in Ehrlich ascites or S180J ascites. In

DISCUSSION

Although the number of tested lysolecithin analogs is insufficient to permit generalizations concerning the structure-activity relationships of these compounds, some features of the dose-response curves obtained in the course of investigation of their effects on the TPCV of Meth A ascites form of Meth A fibrosarcoma.

Male CB6F, mice (5 mice/group) were inoculated i.p. with various numbers of ascites cells; immediately thereafter, the mice were given i.p. injections of lysolecithin in various doses, such that a constant dose of 1 mg/1 x 10^6 ascitic cells was maintained. Diluent was phosphate-buffered 0.85% NaCl solution.

Table 3

<table>
<thead>
<tr>
<th>No. of ascites cells inoculated (x10^6)</th>
<th>MSTa</th>
<th>Range (days)</th>
<th>mg/mouse</th>
<th>MST</th>
<th>Range</th>
<th>T/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>13</td>
<td>13-17</td>
<td>2.0</td>
<td>21</td>
<td>19-24</td>
<td>1.62a</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>13-15</td>
<td>1.0</td>
<td>22</td>
<td>18-27</td>
<td>1.57a</td>
</tr>
<tr>
<td>0.5</td>
<td>14</td>
<td>13-17</td>
<td>0.5</td>
<td>26</td>
<td>24-31</td>
<td>1.86a</td>
</tr>
<tr>
<td>0.25</td>
<td>18</td>
<td>13-24</td>
<td>0.25</td>
<td>21</td>
<td>17-33</td>
<td>1.17</td>
</tr>
</tbody>
</table>

a MST, median survival time.

b T/C ratio of MST's is statistically significant (p < 0.05), as compared to accumulated results of 50 groups of 5 mice each (diluent controls).
Effect of lysolecithin, its analog, and 2 detergents on ascites cells of Meth A fibrosarcoma in vitro

Washed Meth A cells were suspended in Earle’s balanced salt solution (1 × 10^6 cells/ml) containing test substances at 2-fold increments of dilution. After 1 hr exposure at room temperature in air, the proportion of cells killed was determined by microscopic examination after trypan blue staining. In parallel, aliquots of suspensions containing 1 × 10^8 cells in 0.2 ml were inoculated i.d. into male CB6F1 mice in groups of 6 mice, which were observed for presence of tumor at 14 days.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Trypan blue test</th>
<th>Tumor-free mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysolecithin (natural)</td>
<td>7.2 ± 1.5</td>
<td>14.2 ± 1.6</td>
</tr>
<tr>
<td>1-Hexadecylpropanediol-3-phosphorylcholine</td>
<td>8.6 ± 1.7</td>
<td>10.0 ± 1.2</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>37.5 ± 1.4</td>
<td>&gt;49</td>
</tr>
<tr>
<td>Hyamine 10-X hydroxide</td>
<td>6.3 ± 1.6</td>
<td>11.2 ± 1.8</td>
</tr>
</tbody>
</table>

*End point of 3 experiments. The geometric mean in each experiment was based on 2-fold increments of concentration.

Among the ether analogs substituted on C-1 of the propanediol moiety, the antitumor activity decreased from the very effective 1-hexadecylpropanediol-3-phosphorylcholine to the inactive 1-decyl analog. The importance of the nature of the substituents on C-1 and C-2 of the glycerol moiety is demonstrated by: (a) effectiveness of DL-1-dioctylglycerol-3-phosphorylcholine comparable to that of the 1-dodecylpropanediol analog; (b) the activity of the DL-1-octadecyl-2-methylglycerol-3-phosphorylcholine with a methoxyl group on C-2; and (c) greater activity of DL-1-hexadecyl-2-benzylglycerol-3-phosphorylcholine compared with the activity of the similar DL-1-octadecyl analog.

The in vitro exposure of Meth A tumor cells to the natural lysolecithin or to 1-hexadecylpropanediol-3-phosphorylcholine in concentrations of 7.2 and 8.8 µg/ml, respectively, killed 50% of the tumor cells as judged by their staining with trypan blue. Tumor cell suspensions exposed to concentrations of 14.2- and 10.0-µg/ml doses, respectively, of these chemicals failed to produce tumors in 50% of mice at 14 days after i.d. inoculation of cell suspension aliquots containing 1 × 10^8 Meth A cells. Similar effects were produced by Hyamine 10-X hydroxide, a cationic detergent, when Meth A tumor cells were exposed to its solutions of 6.3 and 11.2 µg/ml, respectively. Sodium dodecyl sulfate, an anionic detergent, caused 50% of Meth A cells to stain with trypan blue when used in a concentration of 37.5 µg/ml and prevented tumor growth in 50% of mice inoculated with tumor cells exposed to more than 49 µg/ml of this chemical. Tween 80, a non-ionic detergent, failed to kill tumor cells in as high a concentration as 3.2 mg/ml. Kay (6) has reported the absence of antitumor activity of Tween 80 against the Ehrlich ascites tumor.

Results presented in Charts 3 and 4 show that the inhibition of tumor growth in vivo could not be caused solely by the direct cytotoxic action of lysolecithin since this substance is quickly metabolized after i.p. administration by peritoneal macrophages (10-12) and 2 days after the i.p. injection of lysolecithin labeled in the fatty acid moiety, no radioactivity could be recovered from the peritoneal cavity. Therefore, the tumor growth inhibition observed in mice pretreated with lysolecithin 4 days before tumor implantation is probably mediated by an activated cell population, most likely macrophages. The idea is supported by the fact that peritoneal cells activated by lysolecithin are able to inhibit the growth of Meth A in vitro (12). If the macrophages are removed by appropriate methods, a normal tumor growth in vitro is restored. Lysolecithin injected immediately after tumor inoculation probably kills the majority, but not all, of the tumor cells, especially after administration of its lower doses. The surviving tumor cells are then possibly destroyed by the stimulated peritoneal cells, the killing capacity of which is increased more than 50-fold following exposure in vitro to DL-1-octadecyl-2-methylglycerol-3-phosphorylcholine.

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