Marked Stimulation of Lymphocyte-mediated Attack on Tumor Cells by Target-directed Liposomes Containing Immune RNA

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ABSTRACT

A marked stimulation of normal guinea pig lymphocytes was obtained by incubating them with liposomes that contained both antibody to lymphocytes to provide “homing” of the vesicles and immune RNA isolated from guinea pigs immunized with syngeneic line 10 hepatocarcinoma cells. Tumor cell cytotoxicity was monitored by a 51Cr release assay. This target cell delivery of immune RNA by liposomes produced a dose-dependent stimulation up to 12 times that achieved by in vitro methods with naked immune RNA.

INTRODUCTION

A promising approach to immunotherapy of human neoplasia is the administration of immune RNA directed to tumor cell-associated antigens (14, 15). Immune RNA may be produced in xenogeneic sources and used to stimulate lymphocytes from tumor-bearing animals to specific tumor cytotoxicity without arousing a concomitant immune response to the major histocompatibility complex antigens (6, 15). Several serious problems limit the practical application of immune RNA for therapy: the rapid degradation of immune RNA by body RNases (14), the need that immune RNA reach and interact with lymphocytes, and the presumed need that immune RNA penetrate to the interior of the lymphocyte. Our previous experiments suggested that presentation of immune RNA entrapped in liposomes could help solve these problems. High-molecular-weight polyribonucleotides such as polyinosinic acid:polycytidylic acid were efficiently entrapped in liposomes and were protected from degradation in vivo (9, 18). Antibody could be incorporated into the phospholipid vesicles so that 10 to 15% of the antigen-combining sites would be available at the surface of the particles (8). This technique of incorporating immunoglobulins into the surface of the phospholipid vesicles was used recently to obtain specific targeting of liposomes to cells (2, 3). Liposomes also can be designed to fuse with the plasma membrane and to empty their contents into the cell cytoplasm. Plasma membrane-fusing liposomes that contained actinomycin D were found to reverse the resistance of a cell line to the drug by overcoming a block in cellular uptake (12). The low efficiency of immune RNA stimulation of lymphocytes could be due to an ineffective internalization of the immune RNA and might be overcome by presenting the immune RNA enclosed in liposomes.

MATERIALS AND METHODS

Liposomes were prepared as described previously (9, 18). Forty-three mg sphingomyelin, 8 mg cholesterol, 3 mg dicetyl phosphate, and 6 mg stearylamine (negative liposomes lacked stearylamine) were dissolved in chloroform:methanol (5:1) and distributed equally for the 3 preparations into 250-ml round-bottom flasks. Solvent was removed on a rotary vacuum evaporator, and the lipid films were resuspended individually in aqueous phase (0.9 ml) containing IgG and immune RNA as indicated in 0.1 M phosphate-buffered 0.9% NaCl (pH 7.2). Preparations containing immune RNA were sonically disrupted for 15 sec, whereas preparations containing actinomycin D were sonically disrupted for 1 min. Each preparation was centrifuged (26,000 × g for 25 min) and resuspended 3 times in phosphate-buffered 0.9% NaCl solution. The final volume was adjusted to 2.0 ml and contained 9 to 10 mg lipids per ml. Liposomes containing actinomycin D were prepared by adding 1.0 mg actinomycin D to the flask before the solvent was evaporated (12, 16).

Lymphocytes were prepared in the following manner. Heparinized peripheral blood and cells from minced spleens and lymph nodes from normal Sewall-Wright strain 2 guinea pigs were centrifuged on a Hypaque:Ficoll gradient (4, 5). The lymphocyte layer was washed 3 times and adjusted to 1 × 10⁷ cells/ml in Roswell Park Memorial Institute Medium 1640 containing 15% heat-inactivated fetal calf serum. The preparations contained >85% lymphocytes.

Target cells were prepared from single-cell suspensions of line 10 (or line 1) tumor cells grown as ascites variants in strain 2 guinea pigs. The cells were incubated in tissue culture medium for 48 hr before labeling. The cells were collected by centrifugation, and 1 × 10⁷ cells were resuspended in 10 ml medium (1 × 10⁷) followed by a 24-hr incubation with 200 to 400 µCi ⁵¹Cr. Excess isotope was removed by 4 washes, and the cell concentration was adjusted to 2 × 10⁶ cells/ml.

Cytotoxicity assays were carried out in microtiter plates in a volume of 0.2 ml (11). At the end of the incubation, plates were centrifuged briefly, and released ⁵¹Cr was determined by counting 0.1 ml of the cell-free supernatant by liquid scintillation or γ counting. All assays were performed in triplicate or quadruplicate. Total releasable ⁵¹Cr was determined by freeze-thawing 4 times after resuspend-
ing the cells in distilled water. Control release values were obtained by incubating target cells with nonstimulated lymphocytes. Cell viability (monitored by the trypan blue exclusion method) was >99% in all cells throughout their manipulations.

% of cytotoxicity = \[
\frac{\text{Test cpm} - \text{control cpm}}{\text{Freeze-thaw cpm} - \text{control cpm}} \times 100
\]

Antibodies to line 10 and line 1 hepatocarcinoma cells and strain 2 lymphocytes were prepared by multiple injections of crude cell extracts in complete Freund's adjuvant with 2 mg Tice Bacillus Calmette-Guérin in New Zealand rabbits (10). Antitumor sera were adsorbed with normal strain 2 guinea pig lung fibroblasts, and antilymphocyte sera were adsorbed withuffy-coat cells isolated from peripheral blood of outbred guinea pigs to remove nonspecific cross-reacting antibodies. IgG fractions were prepared by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose. For uptake studies the IgG was iodinated with \(^{125}\)I as described (8). Cross-reactivities of anti-line 10 and anti-line 1 immunoglobulins were measured by determining the binding of \(^{125}\)I-labeled IgG to each tumor cell line. Anti-line 10 preparations were found to bind to line 1 cells 7.7% as efficiently as they bound to line 10, and anti-line 1 preparations bound to line 10 cells 11% as well as they bound to line 1 cells. The antibodies were not cytotoxic.

Specific immune RNA was isolated from spleens and lymph nodes of strain 2 guinea pigs immunized with line 10 or line 1 tumor cells in the presence of Bacillus Calmette-Guérin as described previously (1, 13, 17).

RESULTS

Stimulation of Normal Lymphocytes for Specific Cytotoxicity to Tumor Cells. Chart 1 shows that incubation of normal strain 2 guinea pig lymphoid cells and positively charged liposomes that contained both anti-line 10 immune RNA and antilymphocyte antibody stimulated the cells to become markedly cytotoxic to line 10 tumor cells. The response was dependent on the concentration of liposomes used in the incubation. Liposomes containing only immune RNA or only antilymphocyte antibody were slightly stimulatory or inactive (final cytotoxicity, <2%). The results of similar experiments in Table 1 show that >60% cytotoxicity could be achieved by increasing the concentration of liposomes to 0.1 ml per 1 x 10^7 lymphocytes. These results should be compared to those of experiments with naked immune RNA, which always give a maximal sensitization of lymphocytes of <5%. Liposomes containing RNA isolated from normal guinea pig liver were incapable of stimulating lymphocytes, demonstrating the specificity of the interaction. The specificity of the interaction to various immune RNA's is under further investigation. Polyinosinic acid:polycytidylic acid containing liposomes prepared with or without antilymphocyte immunoglobulin stimulated lymphocytes to a low although significant extent (final cytotoxicity, 1.1 to 16%).

Target-directed Uptake of Liposomes by Lymphoid

<table>
<thead>
<tr>
<th>Liposome contents</th>
<th>Volume of liposomes (µl)</th>
<th>cpm released</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune RNA to line 10 + antilymphocyte IgG¹</td>
<td>25</td>
<td>720 ± 21</td>
<td>2.8</td>
</tr>
<tr>
<td>Immune RNA to line 10 + antilymphocyte IgG</td>
<td>50</td>
<td>6,372 ± 522</td>
<td>53.7</td>
</tr>
<tr>
<td>Immune RNA to line 10 + antilymphocyte IgG</td>
<td>100</td>
<td>7,150 ± 244</td>
<td>60.7</td>
</tr>
<tr>
<td>Antilymphocyte IgG</td>
<td>100</td>
<td>354 ± 10</td>
<td>0</td>
</tr>
<tr>
<td>Immune RNA to line 10</td>
<td>100</td>
<td>373 ± 14</td>
<td>0</td>
</tr>
<tr>
<td>Nonspecific RNA + antilymphocyte IgG</td>
<td>100</td>
<td>422 ± 24</td>
<td>0.1</td>
</tr>
<tr>
<td>Poly(l):poly(C)⁴</td>
<td>100</td>
<td>536 ± 45</td>
<td>1.1</td>
</tr>
<tr>
<td>Poly(l):poly(C) + antilymphocyte IgG</td>
<td>100</td>
<td>587 ± 114</td>
<td>1.6</td>
</tr>
</tbody>
</table>

¹ The aqueous phase contained immune RNA at 800 µg/ml and antilymphocyte IgG at 800 µg/ml.  
² Poly(l):poly(C), polyinosinic acid:polycytidylic acid.  
³ The aqueous phase contained poly(l):poly(C) at 1000 µg/ml and antilymphocyte IgG at 800 µg/ml.

Cells. Radioactive liposomes were prepared with an aqueous phase containing immune RNA and various levels of antilymphocyte IgG. Aliquots were incubated with guinea pig lymphocytes for 18 hr, and the amount taken up by cells was determined (Table 2). The percentage uptake by lymphocytes was dependent on the level of antilymphocyte antibody in the vesicles at the lower concentration of
Table 2
Effect of antilymphocyte IgG concentration in liposomes on uptake of liposomes by lymphocytes

<table>
<thead>
<tr>
<th>Concentration of IgG in aqueous phase (µg)</th>
<th>50 µl</th>
<th>100 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.6</td>
<td>72.7</td>
</tr>
<tr>
<td>160</td>
<td>23.8</td>
<td>68.4</td>
</tr>
<tr>
<td>800</td>
<td>45.8</td>
<td>69.6</td>
</tr>
</tbody>
</table>

Positively charged liposomes containing 14C-labeled cholesterol were prepared by using an aqueous phase containing immune RNA (1.5 mg/ml) and the indicated amounts of antilymphocyte IgG. Lymphocytes (1 x 10^7 cells in 1.0 ml) were incubated with liposomes for 16 hr at 37°C and washed 3 times by centrifugation at 50 x g. The cells were dissolved in 1.0 ml Soluene 350 and counted in 10 ml Dimilume (Packard Instrument Co., Inc., Downers Grove, Ill.).

Cell Killing of Tumor Cells by Target-directed Liposomes Containing Actinomycin D. Additional experiments were carried out to demonstrate that the interaction of target-directed liposomes with cells was dependent on the specificity of the antibody incorporated into the particles. Chart 2 shows that highly cytotoxic liposomes resulted when both actinomycin D and anti-line 10 IgG were entrapped in negatively charged liposomes. The response was dose dependent and resulted in a release of >65% intracellular 51Cr when 25 µl of the preparations were incubated with line 10 tumor cells. These vesicles were 9 times less cytotoxic for line 1 cells (Chart 3). Similarly, liposomes directed against line 1 cells and containing actinomycin D were highly cytotoxic for line 1 cells although somewhat greater cross-reactivity was evident in this case. This agrees with cell binding data that anti-line 1 IgG also showed the greatest amount of cross-reactivity (see "Materials and Methods"). Liposomes that contained only entrapped antibody were not cytotoxic at the highest concentration tested (100 µl), and liposomes containing only actinomycin D also did not interact with the cells sufficiently to cause a release of 51Cr.

DISCUSSION

Positively charged, multilamellar liposomes were selected for these studies with immune RNA because our previous experiments showed that up to 2 mg polyribonucleotides per 20 mg mixed lipids could be incorporated (7) due to electrostatic interactions and trapping of the polymer in the enclosed aqueous phase. <5% of the trapped polyribonucleotide was sensitive to pancreatic RNase, showing that most of the material was "latent" or enclosed within the phospholipid vesicle (7). Our present experiments demonstrate that target-directed liposomes are very effective in vitro carriers for immune RNA. Naked immune RNA alone stimulated lymphocytes to only 0.5 to 5% cytotoxicity in the 51Cr release assay, compared to a maximum of 61% obtained for immune RNA delivered by lymphocyte-directed liposomes.

Negatively charged particles prepared without any antibody are not taken up by cells as readily as are positively charged liposomes (8, 18). Incorporation of antitumor antibody into the negatively charged liposomes and comparison of resulting cytotoxicity to that obtained from non-antibody-containing negative liposomes illustrated the highly specific nature of the antibody-targeting mechanism. Drug cytotoxicity was very closely related to the specificity of the antibody used.

Positively charged, multilamellar liposomes attach readily to cells (8). Specific "homing" of antibody-containing positively charged liposomes was masked by increasing the liposome:lymphocyte ratio; however, the homing effect was clearly evident at lower liposome concentrations. On the
other hand the greatest liposome concentration tested (100 μl) produced the highest lymphocyte stimulation.

The composition and thus the properties of liposomes may be easily varied over a wide range. Consequently, this system is excellent for studying mechanisms for the stimulation of lymphocytes by immune RNA. Especially suited for investigation are the questions of specific interaction with cell surface receptors, cell particle fusion versus phagocytosis, and the involvement of more than 1 cell type in the immune response. Attachment alone may not be sufficient to trigger lymphocyte stimulation, and the requirement for antilymphocyte immunoglobulin may reflect a complex role for additional cell signals or antibody in the immunological process. Our experiments would suggest an intracellular mechanism for immune RNA action. Regardless of the mechanism, target-directed liposomes provide a highly efficient delivery system for immune RNA in a form that is protected from RNase action and is markedly effective in stimulation of lymphocytes.

REFERENCES

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