ABSTRACT

Various vesicle structures and lipid compositions have been studied to identify the optimal type of liposome for delivery of the macrophage-activating agent muramyl dipeptide (MDP) to macrophages. Evaluation of the ability of liposomes to be phagocytosed by macrophages established that optimal initial rates of engulfment were obtained when multilamellar vesicles (MLV) composed of distearoylphosphatidylcholine (18:0 PC):phosphatidylserine (PS) (7:3 mol ratio) were used. MLV composed exclusively of 18:0 PC were phagocytosed at rates > those of MLV composed of egg phosphatidylcholine (PC):PS, whereas MLV composed only of egg PC were very poorly phagocytosed. Although phagocytosis was enhanced by incorporation of PS into MLV, the inclusion of PS brought about significant enhancement in liposome permeability in the presence of serum. The inclusion of PS, however, was a requirement for the delivery of MLV to the lungs following i.v. injection into mice whether used in conjunction with 18:0 PC or egg PC.

Activation of macrophages to become tumoricidal against syngeneic tumor cells with liposome-encapsulated MDP was superior in both degree and duration when MLV composed of 18:0 PC:PS (7:3 mol ratio) were used. MLV were found to be superior to large unilamellar vesicles containing equal amounts of lipid and entrapped MDP. On the other hand, higher levels of macrophage activation were obtained when an equivalent amount of a lipophilic MDP derivative, muramyl tripeptide:phosphatidylethanolamine, was incorporated into the liposome bilayer irrespective of whether the adjuvant was incorporated in liposomes composed of 18:0 PC:PS or egg PC:PS.

INTRODUCTION

An important feature of host defense against infection and neoplastic disease involves the activation of cells of the macrophage-histiocyte series to the cytotoxic state (2, 8, 16). Macrophages that show essentially no cytotoxic activity can be rendered tumoricidal following their interaction with a variety of agents such as lymphokines released by antigen- or mitogen-stimulated lymphocytes, double-stranded RNA, or a variety of microorganisms or their derivatives (5, 6, 10, 11, 17). One such synthetic derivative of bacteria, MDP, is the minimal structure unit with immune-potentiating activity that can replace Mycobacteria in complete Freund's adjuvant (9, 21, 22). MDP influences a variety of macrophage functions such as the production of prostaglandins and collagenase (38), motility (1, 41), enhanced O2-generating capacity (24), proliferation in response to lymphokines (15), and increased cytolytic activity against tumor cells (35).

It has been shown recently that MDP encapsulated within MLV can render rat AM tumoricidal in vitro far more effectively than free nonencapsulated MDP (32). Moreover, systemic administration of MLV-encapsulated MDP resulted in the in situ activation of murine AM which was associated with eradication of spontaneous pulmonary and lymph node metastases (14).

These studies and others (13, 27, 28) have demonstrated that the size and lipid composition of the vesicles used are very important in (a) the efficient uptake of liposomes by macrophages and (b) the arrest and retention of liposomes in the lung, a major site of metastatic disease (12). However, the inherent leakage properties and in situ stability of liposomes may be crucial for long-term maintenance of macrophages in the tumoricidal state. It is indeed possible that, following phagocytosis, the liposomes act as a depot by slowly sustaining intracellular release of encapsulated activating agents in situ. In an effort to explore this possibility and delineate the optimal types of carrier liposomes, we have analyzed the uptake and activation of macrophages with liposomes of various types and lipid compositions containing either encapsulated MDP or MTP-PE inserted directly into the liposome phospholipid bilayer.

MATERIALS AND METHODS

Animals. Specific pathogen-free male inbred F344 rats, 8 to 10 weeks old, and 6-week-old C57BL/6 mice were obtained from the Animal Production Area of the NCI/Frederick Cancer Research Facility.

Media and Reagents. All monolayer cultures were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine (Flow Laboratories, Inc., Rockville, Md.). All cultures were incubated at 37° in a humidified atmosphere containing 5% CO2.

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Muramyl Dipeptide1

of the Tumoricidal Properties of Macrophages by Liposomes Containing

Muramyl Dipeptide1

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1 The abbreviations used are: MDP, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine with dipalmitoyl phosphatidylethanolamine in the presence of dicyclohexylcarbodiimide. The MDP preparations were free of endotoxins as determined by the Limulus amoebocyte lysate sn-glycerol-3'-phosphate(lithium)amide (muramyl tripeptide phosphatidylethanolamine); CF, carboxyfluorescein; PC, phosphatidylcholine; 18:0 PC, distearoylphosphatidylcholine; PS, phosphatidylserine (beef brain); NBD-PE, 4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; PBS, phosphate-buffered saline (0.15 mol NaCl/0.01 mol KH2PO4, Na2HPO4); REV, reverse-phase evaporation vesicles; BSA, bovine serum albumin; Tc, transition temperature.
assay. CF was purchased from Eastman Kodak Co., Rochester, N. Y., and purified as described by Blumenthal et al. (3).

Preparation and Purification of AM Cultures. Rat or mouse AM were obtained by tracheobronchial lavage and characterized as described elsewhere (31). The lavaged cellular suspension was washed, and 10^6 AM (>96%) were plated into wells of a Microtest II plate (Falcon Plastics, Oxnard, Calif.). Nonadherent cells (<10%) were removed by washing with supplemented Eagle's minimal essential medium 60 min after the initial plating. Practically all of the adherent cell population had mononuclear morphology, phagocytosed carbon particles, and opsonized sheep RBC.

Lipids and Preparation of Liposomes. Chromatographically pure egg PC, dimyristoylphosphatidylethanolamine, 18:0 PC, and PS, isolated from beef brain, were purchased from Avanti Biochemicals, Birmingham, Ala. Unless specifically stated otherwise, PC-PS were admixed at a 7:3 molar ratio. The fluorescent lipid, NBD-PE, was prepared by reacting N-4-nitrobenz-2-oxa-1,3-diazole-chloride with dimyristoylphosphatidylethanolamine and purified by silicic acid chromatography as described previously (23). The product was further purified by preparative thin-layer chromatography and exhibited a single fluorescent spot. MLV were prepared as follows and used immediately. Appropriate lipids were mixed in chloroform, dried under nitrogen, and further dried in a vacuum. The lipids were then hydrated in PBS or PBS containing MDP or CF and mechanically vortexed at room temperature or at 60°C for egg PC and 18:0 PC MLV, respectively. Integration of MTP-PE into lipid bilayer was verified by the ability of anti-MDP antibodies to specifically precipitate MTP-PE MLV. REV were prepared by the method of Szoka and Papahadjopoulos (34) from egg PC-PS only. The MLV were immediately chilled to 2°C and repeatedly washed in centrifugation (15,000 × g for 10 min). The MLV preparations were then adjusted to 1 μmol lipid per ml and were calculated to have entrapped approximately 2.5 μg aqueous phase per μmol lipid. MLV were added to macrophage cultures at a dose of 100 nmol lipid per 10^5 cells.

Phagocytosis of Liposomes. Macrophage binding and phagocytosis of liposomes were assessed by incubating AM monolayers with liposomes (100 nmol lipid per 10^6 AM) containing 0.1% NBD-PE for different periods of time with or without 10^-4 M sodium azide. The monolayers were then extensively washed with PBS, and the cells were lysed with 1% sodium dodecyl sulfate. An aliquot of the lysate was removed, and the lipids were extracted with chloroform:methanol:water (1:2:0.8). Insoluble material was removed by centrifugation and reextracted with the same solvent. One volume each of chloroform and water was added to the extracts, and the mixture was chilled and centrifuged to separate the aqueous and organic phases. The chloroform phase was removed and dried under a stream of nitrogen. The residue was dissolved in chloroform:methanol (1:9), and the relative fluorescence of the extracts was determined.

Organ Localization and Retention of Liposomes Administered i.v. Liposomes containing entrapped 125I-BSA were injected i.v. into C57BL/6 mice (5/group). Organ localization and retention of liposomes were determined as described previously (13).

Leakage of CF from MLV. In order to assess the extent of leakage of low-molecular-weight materials from MLV, liposomes were prepared by disrupting the dry lipid film in the presence of self-quenching concentrations (200 mM) of CF (3, 39). These experiments are based on the phenomenon of “self-quenching” commonly seen with many fluorophores. Briefly, the fluorescence of CF is proportional to its concentration, but as its concentration is raised above ~10 μM, the fluorescence yield per molecule rapidly drops because of interaction between fluorophores. Thus, a suspension of MLV containing 200 mM CF entrapped in its aqueous compartment fluoresces only slightly. However, intense fluorescence is observed when CF leaks (and subsequently dilutes) into the medium or when MLV are lysed by detergent. Self-quenching thus allows material remaining in intact MLV to be distinguished from that which has leaked into the medium. In the present study, leakage of CF from vesicles was calculated by measuring the spontaneous release of the dye from self-quenched vesicles (Frect) and the total amount of CF in the test system after the addition of Triton X-100, which induces complete vesicle lysis (Fmax). The quantitative CF leakage data were analyzed as described by Van Renswoude et al. (37).

Fluorometry. Continuous monitoring of CF leakage from vesicles was carried out with a Farrand MK II spectrophotofluorometer equipped with a water-jacketed cuvet and strip chart recorder. Bleaching of fluorescence by the exciting light was kept to a minimum (<1%/hr) by using narrow (1 nm) band pass slits. Excitation and emission wave-lengths were 490 to 520 nm and 470 to 525 nm for CF and NBD-PE, respectively.

Assay of AM-mediated Cytotoxicity in Vitro. AM-mediated cytotoxicity was assessed by a radioactive release assay detailed previously (31). Target cells in exponential growth phase were incubated for 24 hr in media containing 0.2 μCi [125I]iododeoxyuridine per ml (specific activity, 200 mCi/mol; New England Nuclear, Boston, Mass.). The cells then were washed in warm Hanks’ balanced salt solution to remove unbound radiolabel, harvested by a short trypsinization (0.25% Difco trypsin:0.2% EDTA for 1 min at 37°C), and resuspended in media; 1 × 10^5 target cells were plated per well to obtain an initial AM:target cell ratio of 10:1. At this population density, normal (untreated) AM are not cytotoxic to neoplastic cells, whereas activated AM are. No significant differences were detected in the plating of [125I]iododeoxyuridine-labeled target cells to the plastic or to monolayers of AM (normal or activated). Radiolabeled target cells also were plated alone as an additional control group. All cultures were washed 4 hr after the plating of tumor cells. The AM-target cell cultures were then incubated in media up to 7 days at 37°C. At various time points after the addition of MLV, cultures were washed twice with Hanks’ balanced salt solution, and the adherent cells were lysed with 0.1 ml of 0.5 N NaOH and washed with Hanks’ balanced salt solution. The lysisate and the washes were pooled, and their radioactivity was measured in a gamma counter.

The percentage of cytotoxicity mediated by activated AM was calculated as follows:

% of cytotoxicity = \( \frac{\text{cpm in target cells cultured with control AM} - \text{cpm in target cells cultured with test AM}}{\text{cpm in target cells cultured with control AM}} \) × 100

The statistical significance of differences between test groups was determined by Student’s 2-tailed t test.

RESULTS

Phagocytosis of MLV by Macrophages. The interaction of neutral (egg PC and 18:0 PC) and negatively charged MLV (PC:PS, 7:3) containing 0.1% NBD-PE with monolayer cultures of macrophages is shown in Charts 1 and 2. The results indicate that the efficient uptake of egg PC MLV is dependent upon the presence of PS, which resulted in ~10-fold higher uptake. On the other hand, MLV composed of 18:0 PC were efficiently phagocytosed by the cells regardless of the presence of PS. Although MLV containing 18:0 PC were phagocytosed as well as, or better than, egg PC MLV containing PS, the addition of PS to 18:0 PC MLV improved uptake by approximately 2-fold (Chart 1).

Most of the fluorescence associated with the cells is attributed to endocytozed lipid, which is directly related to the binding of MLV to the cell surface. The results presented in Chart 2 show that the addition of PS resulted in significantly higher binding of MLV to the macrophage surface than with PC alone. As was the case with phagocytosis, the extent of binding of MLV to the cell surface was as follows: 18:0 PC:PS > 18:0 PC > egg PC:PC > egg PC.
Effects of Liposome Structure and Composition on Macrophage Activation

Examination of washed macrophages incubated for 2 hr with 18:0 PC:PS MLV containing 0.1% MBD-PE revealed that more than 90% of the cells phagocytosed MLV (Fig. 1). No significant fluorescence was detected at the surface of the treated cells, suggesting that, after initial MLV cell binding, phagocytosis ensues very rapidly. Moreover, a diffuse distribution of NBD-PE within the cytoplasm was never seen, suggesting that dissolution of endocytosed MLV occurs at a very slow rate. Indeed, freeze-fracture analysis of macrophages containing endocytosed MLV revealed what appeared to be intact liposomes (Fig. 2).

Leakage of Low-Molecular-Weight Solute from MLV. MLV prepared by vortexing the dried lipid films in 200 mmol of CF were extensively washed with ice-cold PBS until a negligible amount of fluorescence was detected in the supernatants. Aliquots of MLV (~50 nmol lipid) were added to a cuvet containing 1 ml medium, and the appearance of fluorescence was monitored by continuous measurements in a spectrophotofluorometer for up to 1 hr ($F_{\text{direct}}$). The total amount of CF within the test system was assessed by the addition of detergent after the 1-hr incubation period ($F_{\text{direct}}$). The percentage of leakage of CF was then calculated as described in "Materials and Methods."

Table 1 shows the percentage of CF released from MLV of differing lipid composition after a 1-hr incubation in the presence of PBS and PBS containing 5% fetal bovine serum. The release of CF from all the MLV compositions tested was similar when incubated in PBS alone (2 to 5%/hr). On the other hand, the addition of PS to either egg PC or 18:0 PC resulted in substantially higher leakage rates when the PBS contained 5% fetal bovine serum. Leakage of CF from 18:0 PC:PS was ~2-fold higher than from egg PC:PS MLV (21 and 11%/hr, respectively).

Localization of MLV Injected i.v. in the Lungs. Since MLV composed of 18:0 PC are more avidly phagocytosed by macrophages than are egg PC MLV, they appear to be a better choice for experimental therapy studies because rapid uptake by peripheral blood monocytes may be a prerequisite for efficient macrophage activation in situ (see "Discussion"). However, since the lungs are a primary site for metastatic disease, it is imperative to determine whether MLV composed of 18:0 PC are localized and retained in the lungs following i.v. administration.

The localization and retention of MLV of different lipid compositions containing entrapped $^{125}$I-BSA within the lungs of C57BL/6 mice after i.v. injection are shown in Table 2. Although 18:0 PC MLV were efficiently phagocytosed by macrophages (Fig. 1), localization in the lung required the presence of PS, as is the case with egg PC MLV (Table 2; Refs. 13 and 20). Localization in the lung was only 2% with MLV composed of 18:0 PC alone, whereas an increase in PS content to 30 mol% resulted in more than 6% retention.

Importance of the Lamellar Structure and Lipid Composition.

Table 1

<table>
<thead>
<tr>
<th>MLV composition</th>
<th>PBS</th>
<th>PBS and 5% fetal bovine serum</th>
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</thead>
<tbody>
<tr>
<td>Egg PC</td>
<td>2.0 ± 1.0</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Egg PC:PS (7:3)</td>
<td>4.9 ± 1.1</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>18:0 PC</td>
<td>3.7 ± 0.9</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>18:0 PC:PS (7:3)</td>
<td>3.2 ± 0.7</td>
<td>21.1 ± 2.6</td>
</tr>
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</table>

* Mean ± S.D. of 3 independent assays.
* Numbers in parentheses, mol ratios of the indicated components.

Table 2

<table>
<thead>
<tr>
<th>MLV composition</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg PC</td>
<td>2.31</td>
<td>18.27</td>
<td>1.21</td>
</tr>
<tr>
<td>Egg PC:PS (7:3)</td>
<td>6.19</td>
<td>16.80</td>
<td>0.91</td>
</tr>
<tr>
<td>18:0 PC</td>
<td>2.03</td>
<td>15.55</td>
<td>1.26</td>
</tr>
<tr>
<td>18:0 PC:PS (9:1)</td>
<td>2.23</td>
<td>18.58</td>
<td>1.75</td>
</tr>
<tr>
<td>18:0 PC:PS (8:2)</td>
<td>2.29</td>
<td>13.13</td>
<td>0.91</td>
</tr>
<tr>
<td>18:0 PC:PS (7:3)</td>
<td>6.58</td>
<td>16.87</td>
<td>1.19</td>
</tr>
</tbody>
</table>

* The results are expressed as the percentage of the total injected dose of MLV.
* Numbers in parentheses, mol ratios of the indicated components.
tion of Liposomes Containing MDP for Activation of Macrophages. If slow sustained release of liposome-encapsulated immunomodulating agents in situ is a prerequisite for prolonged duration of its immunopotentiating effects, the number of lamellae present in the liposome structure as well as the lipid composition may be important. In order to clarify this point, normal rat AM were incubated with 2 different liposome types, MLV (>5 lamellae) and REV (<5 lamellae), adjusted to contain equal amounts of lipid and entrapped MDP. Relative capture volumes of the various vesicle types were assessed independently by determining the extent of $^{125}$I-BSA and CF entrapment. Incubation of normal rat AM for 24 hr with liposome-encapsulated MDP, 100 nmol lipid containing 0.25 μg MDP (0.5 nmol) per $10^6$ cells, rendered them tumoricidal against syngeneic MADB-200 adenocarcinoma cells. Normal syngeneic embryo cells were unharmed (data not shown). Control populations of AM incubated with media or with liposomes and unencapsulated MDP were not tumoricidal (Table 3). Significant cytotoxicity was initially evident with all the liposome preparations tested; however, the extent of tumoricidal activity and its duration were clearly dependent on the type and composition of the vesicles. MLV composed of 18:0 PC:PS activated AM to a greater extent, and significant cytotoxicity was clearly evident for 7 days after initial treatment of macrophages. The duration of significant cytotoxicity was similar for both MLV and REV composed of egg PC:PS, although MLV were clearly better inducers of cytotoxicity for the first 72 hr.

Activation of AM by MTP-PE MLV. The foregoing results demonstrate the importance of liposome structure and composition for the activation of macrophages using water-soluble MDP entrapped in the aqueous compartment of liposomes. However, if a lipid MDP derivative were intercalated in the vesicle bilayer, it is possible that a lipid MDP composition would be inconsequential to the degree of activation since vesicle leakage properties would be unimportant. In order to examine this possibility, MTP-PE was incorporated into MLV composed of 18:0 PC:PS or egg PC:PS (7:3:0.4 mol ratio). The intercalation of MTP-PE into the lipid bilayer was assessed by the specific precipitation of MLV with anti-MDP antibodies as described in "Materials and Methods." Incubation of normal rat AM for 24 hr with liposomes, 100 nmol lipid containing 0.4 nmol MTP-PE, rendered them tumoricidal against syngeneic MADB-200 adenocarcinoma target cells (Table 4). The levels of cytotoxicity obtained with MTP-PE were consistently higher than those obtained with MDP entrapped in egg PC:PS MLV. In contrast to MLV containing encapsulated MDP, MLV composed of 18:0 PC:PS or egg PC:PS and containing MTP-PE were equally effective in both the extent and duration of macrophage activation.

**DISCUSSION**

The present study provides a detailed analysis of the uptake

<table>
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<tr>
<th>Macrophage treatment</th>
<th>AM-mediated cytotoxicity on</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, media control</td>
<td>2131 ± 415*</td>
</tr>
<tr>
<td>MLV (18:0 PC:PS)</td>
<td>1522 ± 341</td>
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<tr>
<td>MLV (egg PC:PS)</td>
<td>1124 ± 41 (47)</td>
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<tr>
<td>REV (egg PC:PS)</td>
<td>1534 ± 87 (28)</td>
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<tr>
<td>REV (egg PC:PS with 5 μg unencapsulated MDP)</td>
<td>1925 ± 337 (10)</td>
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<tr>
<td>MLV (egg PC:PS with 5 μg unencapsulated MDP)</td>
<td>2033 ± 413 (5)</td>
</tr>
<tr>
<td>MLV (18:0 PC:PS with 5 μg unencapsulated MDP)</td>
<td>2125 ± 279 (&lt;1)</td>
</tr>
</tbody>
</table>

*Macrophages ($10^6$) were plated into 38-mm culture wells and incubated with liposomes [PC:PS (7:3 mol ratio); 100 nmol total lipid] for 24 hr. The cultures were then washed, and $10^6$ syngeneic $[^{125}]$iododeoxyuridine-labeled MADB-200 target cells were added for 72 hr.

**DISCUSSION**

The present study provides a detailed analysis of the uptake

<table>
<thead>
<tr>
<th>AM treatment</th>
<th>AM-mediated cytotoxicity on</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, media control</td>
<td>1153 ± 41*</td>
</tr>
<tr>
<td>18:0 PC:PS:MTPE-PE (7:3:0.4)</td>
<td>1099 ± 108</td>
</tr>
<tr>
<td>Egg PC:PS:MTPE-PE (7:3:0.4)</td>
<td>152 ± 30 (89)*</td>
</tr>
<tr>
<td>18:0 PC:PS (7:3)</td>
<td>1208 ± 90</td>
</tr>
<tr>
<td>Egg PC:PS (7:3)</td>
<td>1186 ± 59</td>
</tr>
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</table>

*Macrophages ($10^6$) were plated into 38-mm culture wells and incubated with liposomes, 100 nmol total lipid, for 24 hr. The cultures were then washed, and $10^6$ syngeneic $[^{125}]$iododeoxyuridine-labeled MADB-200 target cells were added for 72 hr.

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of liposomes by macrophages and their subsequent activation to become tumoricidal. Liposomes of different physical structures and lipid compositions containing the immunopotentiating agent MDP were studied. The results presented here and in previous studies strongly suggest that phagocytosis is the dominant mode of vesicle uptake by macrophages (28) and that the extent of binding and subsequent phagocytosis are dependent upon MLV lipid composition. The phagocytosis of MLV composed exclusively of neutral lipids (PC) is directly associated with the $T_c$ of the lipids used. Egg PC MLV ($T_c < 37^\circ$) do not readily bind nor are they avidly phagocytosed by macrophages. However, 18:0 PC MLV ($T_c > 37^\circ$) bind and are phagocytosed at a significantly faster rate. These results are in agreement with the general observations that lipids below the gel-liquid crystalline $T_c$ adsorb to cell surfaces, whereas lipids above $T_c$ become cell associated predominantly by other pathways (for reviews, see Refs. 25 and 26). On the other hand, agreement with the general observations that lipids below the gel-liquid crystalline $T_c$ adsorb to cell surfaces, whereas lipids above $T_c$ become cell associated predominantly by other pathways (for reviews, see Refs. 25 and 26). On the other hand, however, when PS is added to MLV, rendering a net negative charge, they become avidly bound to the cells and are subsequently rapidly phagocytosed irrespective of the bulk phospholipid composition.

Although the lipid $T_c$ of PC MLV is important for their efficient phagocytosis by macrophages, it has no effect on their ultimate arrest and retention in the lungs following i.v. administration. The observation that MLV containing PS are arrested in the lungs more efficiently than are neutral MLV agrees with the earlier findings of Kimelberg (20) and Fidler et al. (13) and suggests that the $T_c$ of the bulk lipid phase does not play a role in the ultimate distribution of liposomes in vivo.

The extent of MDP leakage from MLV was estimated by assaying the release of CF at self-quenching concentrations from MLV. It should be noted, however, that the absolute rates of MDP and CF leakage may be different due to charge differences between these 2 compounds. We therefore use these data only as an indication of the relative leakiness of the different types of vesicles used. The results indicate that MLV composed of either egg or 18:0 PC are relatively resistant to leakage in the presence or absence of serum at $37^\circ$ (2 to 5% leakage per hr). However, the inclusion of PS brought about significant enhancement in MLV permeability in the presence of serum.

Recent attention has focused on the use of liposomes as carrier vehicles for the transport of active materials into target cells in vitro and in vivo. Our data demonstrate that MDP encapsulated within MLV composed of PC:PS could generate macrophage tumoricidal activity to a significantly higher degree than REV prepared from the same lipids, whereas free unencapsulated MDP at the same dosage had virtually no effect. Moreover, MLV composed of 18:0 PC were superior to egg PC MLV for both the extent and duration of macrophage activation. Significant tumoricidal activity was clearly evident 7 days after the initial incubation of macrophages with 18:0 PC:PS MLV, whereas at that time, both egg PC:PS MLV and REV sustained negligible levels of cytotoxicity. Significant differences in the ability of 2 different MLV to evoke macrophage tumoricidal activity, however, were not evident when a lipophilic derivative of MDP, MTP-PE, was inserted into the liposomal membranes. Egg PC:PS MLV and 18:0 PC:PS MLV both activated macrophages to a similar degree.

It was surprising that, in the presence of serum, 18:0 PC:PS MLV, which are superior inducers of macrophage tumoricidal activity, leak at an ~2-fold faster rate than do egg PC:PS MLV.

We can only speculate on the reasons for enhanced leakage from MLV containing CF. The binding and subsequent phagocytosis of PC:PS liposomes by macrophages are unaffected by the presence of serum, whereas liposomes composed exclusively of PC are inhibited ~4-fold when serum is added to the culture media. MLV can readily associate with certain proteins (18, 19, 36, 42), and such an association may lead to enhanced solute leakage from the MLV interior (36, 42). Moreover, certain cell surface proteins are transferred to vesicles during vesicle-cell incubations (4, 7), and the transfer of egg PC to serum components can result in the inhibition of MLV uptake by cells (18, 30). It is conceivable that an isothermal phase separation within the MLV membrane ultimately could lead to the observed leakage. Such a phase separation could exist by virtue of the MLV lipid composition which influences the interaction between the MLV bilayer and serum components. Indeed, a preexisting phase separation may facilitate the interaction of the MLV bilayer with high-density lipoproteins (29, 33, 40). In any event, it is possible that the presence of PS in liposomes in combination with 18:0 PC, which results in rapid binding and phagocytosis by macrophages, circumvents problems of leakage into the extracellular medium. Once endocytosed, MLV may function as a "slow release depot" in which encapsulated material is released over a longer period of time (13). Furthermore, 18:0 PC:PS MLV may be more resistant to intracellular lysosomal degradation than MLV PC:PS and thus produce a sustained activation of macrophages.

In conclusion, we have demonstrated that the extent and duration of macrophage activation by liposomes containing MDP are dependent on the physical structure and lipid composition of the vesicles. For this reason, it is possible that, by manipulating these factors, a more effective and yet less intensive regimen could be developed for the systemic activation of macrophages for the purpose of achieving the regression of spontaneous metastases (14).

ACKNOWLEDGMENTS

We thank William E. Fogler and Debra Smith for their excellent technical assistance.

REFERENCES


* A. J. Schrott and I. J. Fidler, unpublished observations.
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Effects of Liposome Structure and Composition on Macrophage Activation

Fig. 1. Phase and fluorescent photomicrographs of macrophages after 2-hr incubation with 18:0 PC:PS:0.1% NBD-PE. Bar, 20 μm.

Fig. 2. Freeze-fracture photomicrograph of a macrophage 24 hr after phagocytosis of 18:0 PC:PS (7:3) MLV. Bar, 1 μm.
Effects of Liposome Structure and Lipid Composition on the Activation of the Tumoricidal Properties of Macrophages by Liposomes Containing Muramyl Dipeptide

Alan J. Schroit and Isaiah J. Fidler