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Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing muramyl dipeptide

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ABSTRACT **The multiple systemic administration of multilamellar liposomes composed of phosphatidylserine and phosphatidylcholine (molar ratio 3:7) that contained water-soluble muramyl dipeptide (MDP) activated alveolar macrophages to become tumoricidal and eradicated established spontaneous pulmonary and lymph node metastases. Spontaneously metastasizing melanoma cells were injected into the footpads of mice. After 4–5 weeks, the tumors were resected by a midfemoral amputation; 3 days later, twice-weekly injections of liposomes were initiated and continued for 4 weeks. In some experiments the mice were killed 2 weeks after the final treatment. Seventy-four percent of animals injected with liposomes containing MDP were free of visible metastases. In a separate life-span experiment, 60% of mice treated with liposome-encapsulated MDP were tumor-free 120 days after the last liposome treatment or 110 days after all control mice treated with free MDP or control liposome preparations had died of disseminated cancer. These data suggest that the systemic administration of liposomes containing MDP, or similar compounds that produce macrophage activation, may provide an additional useful approach to the therapeutic regimens currently used to eradicate cancer metastases.**

Metastasis of malignant neoplasms is responsible for most therapeutic failures in clinical oncology (1–3). Recent studies suggested that metastases can result from the proliferation of a minor subpopulation of cells within the primary tumor and that tumors can be heterogeneous with regard to many phenotypic characteristics such as drug sensititivity and metastatic potential (1–3). Such biological diversity among metastases implies that successful therapy of disseminated disease must include a regimen that acts by a mechanism independent of this heterogeneity.

One biological agent that appears to function against tumor cells without regard to their phenotypic diversity is the activated macrophage. At least *in vitro*, macrophages can distinguish tumorigenic from nontumorigenic cells by a mechanism that is independent of such phenotypic characteristics as drug sensitivity, metastatic potential, and antigenicity (4). Moreover, to date, attempts to select *in vitro* tumor cells resistant to macrophage-mediated cytotoxicity have been unsuccessful (5).

The increasing evidence that macrophages are important in host defense against neoplasia has stimulated interest in agents that can enhance macrophage-mediated destruction of tumor cells. Normal macrophages can be activated to become tumoricidal by various agents such as lymphokines and by whole microorganisms or their products such as endotoxins (6). However, the use of whole viable microorganisms or their products to activate macrophages *in vivo* has been hampered by a number of undesirable side effects (7).

The search for synthetic compounds that are relatively nontoxic yet possess immune potentiating activities has resulted in the demonstration that *N*-acetylmuramyl-L-alanyl-D-isoglutamine [muramyl dipeptide. (MDP), M_r 492] is the minimal structural unit with immune potentiating activity that can replace mycobacteria in Freund's complete adjuvant (8–10). MDP is known to influence many macrophage functions such as the production of prostaglandins and collagenase (11), motility (12), enhanced O_2 generating capacity (13), proliferation in response to lymphokines (14), cytolytic activity (15), and production of lymphocyte-activating factors (16). Moreover, MDP encapsulated within liposomes—concentric phospholipid vesicles separated by aqueous compartments—can render rat alveolar macrophages (AM) tumoricidal *in vitro* (17).

Recently, the successful treatment of metastases of the B16 melanoma in C57BL/6 mice and of the K-1735 melanoma in C3H mice by intravenous injection of multilamellar vesicles (MLV), liposomes, containing crude preparations of lymphokines was reported (18). Although this approach is attractive, there are several disadvantages associated with the use of crude lymphokine preparations for this purpose. In common with many biological materials, lymphokine preparations can vary in their potency, composition, and biological efficacy. In contrast, MDP is a small synthetic molecule that can be obtained in a pure form, and its activity can be quantitated *in vitro* or *in vivo*. Because MDP encapsulated within MLV is far more efficient in activating macrophages *in vitro* than is free (water-soluble) MDP (17), we wished to determine whether MLV-encapsulated MDP could efficiently activate macrophages *in vivo* and provide a mechanism for the destruction of spontaneous lymph node and visceral metastases.

MATERIALS AND METHODS

Animals. Specific-pathogen-free mice of the inbred strain C57BL/6N and F344 strain rats were obtained from the Frederick Cancer Research Center's Animal Production Area.

Cell Cultures. The B16-BL6 variant line of the C57BL/6 melanoma has been adapted to grow *in vitro*. After implantation at a subcutaneous site, this tumor metastasizes to the lungs and lymph nodes in about 90% of mice (19). Monolayer cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air and maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine. The components of this medium, designated CME medium,

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Abbreviations: MDP, muramyl dipeptide; AM, alveolar macrophages; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; MLV, multilamellar vesicles (liposomes); CME medium, complete Eagle's minimal essential medium; DB saline, Dulbecco's Ca^{2+}- Mg^{2+}-free physiologic basic salt solution.

were obtained from Flow Laboratories (McLean, VA). All cultures were free of *Mycoplasma* and the following pathogenic murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's encephalitis virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (MA Bioproducts, Walkersville, MD).

Preparation and Purification of AM Cultures. Mouse AM were harvested by a tracheobronchial lavage method (20). The lavaged AM suspension was centrifuged and resuspended; 10^5 AM were plated into wells of a Microtest II plate with a surface area of 38 mm^2 (Falcon). Nonadherent cells (less than 10%) were removed by washing with CME medium 60 min after initial plating. At that time, practically all (>98%) of the adherent cells were mononuclear, phagocytosed carbon particles, and opsonized sheep erythrocytes.

Lipids and Preparation of Liposomes. Chromatographically pure egg phosphatidylcholine (PtdCho) and beef brain phosphatidylserine (PtdSer) were purchased from Avanti Biochemicals (Birmingham, AL). MLV were prepared from a mixture of PtdCho and PtdSer (mole ratio, 7:3) mechanically agitated on a Vortex mixer as described (21). Encapsulation of MDP or CME medium within liposomes was achieved by using methods similar to those described (20, 21). We used PtdSer/PtdCho MLV as carriers for MDP because they are not toxic at the dose used here (22) and are arrested efficiently in the lungs (in addition to organs of the reticuloendothelial system) after intravenous administration (23). MLV preparations were always used within 4 hr. For *in vitro* activation, MLV containing encapsulated aqueous material were added to macrophages at a concentration of 100 or 200 nmol of phospholipid per 10^5 cells. This dose of MLV contained approximately 0.5 μl of encapsulated material [Dulbecco's Ca^{2+}- Mg^{2+}-free physiologic basic salt solution (DB saline) or MDP] and should be compared with the 200 μl (volume of the culture well) used to induce macrophage activation with free MDP. The amount of free MDP and the dose of MLV used routinely to render AM tumoricidal were nontoxic to target cells as determined in pilot experiments.

***In Vitro* Activation of AM by Free MDP and MLV-Encapsulated MDP.** MDP was obtained from Calbiochem. The MDP did not contain endotoxins (lipopolysaccharide) as detected by the *Limulus* lysate assay. AM were incubated in CME medium alone, CME medium containing free MDP, or in CME medium containing MLV-encapsulated MDP for up to 24 hr at 37°C. Controls always included MLV containing DB saline and suspended in free MDP. The AM cultures were rinsed thoroughly with CME medium before the addition of target cells.

Activation of AM After Intravenous Injection of Free Or MLV-Encapsulated MDP. Mice were injected intravenously with either DB saline, free MDP (200 μg per mouse), or MLV (10 μmol of phospholipids) containing either MDP (2.5 μg) or DB saline (empty liposomes). The MLV were suspended in DB saline and inoculated in a vol of 0.2 ml. Empty liposomes in DB saline containing free MDP (2.5 μg per mouse) were also injected. Mice were killed at 4, 24 or 48 hr after injection. AM were harvested by lavage with DB saline as described above.

Macrophage-Mediated Cytotoxicity Assay. Macrophage-mediated cytotoxicity was assessed by a radioactive release assay as described (24). After an activation period *in vitro* (or *in vivo*), the cultures were washed with CME medium, and 5×10^3 [^{125}I]IUdR-labeled target cells were added to each well in 0.2 ml of CME medium. Target cells alone were always plated as an additional control. Twenty-four hours after the addition of target cells, the cultures were washed to remove nonplated cells and re-fed. At 24 or 72 hr after plating, adherent target cells were lysed with 0.1 ml of 0.2 M NaOH. The lysate was adsorbed on cotton swabs which were assayed for radioactivity in a gamma counter. The percentage cytotoxicity in the macrophage assays was computed by:

$$\frac{\text{cpm of target cells with normal macrophages} - \text{cpm of target cells with activated macrophages}}{\text{cpm of target cells with normal macrophages}} \times 100.$$

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

Regression of Spontaneous Metastases After Intravenous Injection of MLV Containing MDP. C57BL/6 mice were injected subcutaneously in the footpad with 2.5×10^4 viable B16-BL6 tumor cells suspended in 0.05 ml of DB saline. Four to 5 weeks later, when the tumors reached 10–15 mm in diameter, the mice were anesthetized by methoxylflurane inhalation and the tumor-bearing leg, including the popliteal lymph node, was amputated at midfemur. MLV treatment was begun 3 days later, and each treatment consisted of a tail vein injection of MLV (5 μmol of phospholipids) suspended in 0.2 ml of DB saline. The MLV consisted of PtdSer and PtdCho at a molar ratio of 3:7 and contained either MDP (2.5 μg per mouse) or DB saline (empty liposomes). DB saline-containing liposomes were suspended in DB saline containing 2.5 μg of MDP per mouse. Additional controls included mice injected intravenously with free MDP (100 μg per mouse) or DB saline. Treatments were given twice weekly for 4 weeks.

In the first set of experiments, mice were killed 2 weeks after the final treatment. Pulmonary metastases in each animal were counted under a dissecting microscope by two independent observers. All suspected pulmonary and extrapulmonary metastases were confirmed by microscopic examination of fixed histological sections. In a second set of experiments, mice were observed daily for 190 days. Dead or moribund mice were necropsied to ascertain the presence or absence of disseminated cancer. Animals surviving at least 120 days after the last treatment were considered to be disease free (25, 26). These animals were then challenged subcutaneously with 2.5×10^4 viable tumor cells to determine whether they could support the growth of the B16 tumor. All animals were killed 2 weeks after the second tumor challenge and necropsied.

RESULTS

Activation of AM by Intravenous Injection of MLV Containing MDP. The *in vitro* cytotoxicity of AM harvested after intravenous treatment of mice was measured against the B16-BL6 tumor cells also used in the *in vivo* assays reported below. Data for a representative experiment (one of three) are shown in Table 1. The intravenous injection of DB saline, free MDP (200 μg per mouse), or empty MLV plus 5 μg of free MDP did not render mouse AM tumoricidal. In contrast, the injection of MLV containing 2.5 μg of MDP per mouse activated AM to become cytotoxic against the B16-BL6 cells. When harvested 4 hr after intravenous injection of MLV-encapsulated MDP, mouse AM were activated to lyse 31% of the B16 cells ($P < 0.01$). By 24 hr after treatment, the AM lysed 38% of the targets ($P < 0.01$). When AM were harvested 48 hr after treatment, their cytotoxicity had diminished to 17% ($P < 0.02$).

Kinetics of AM Activation *in Vitro* with Free and MLV-Encapsulated MDP. The above experiments demonstrated that free MDP, even at 80–100 times the dose encapsulated within MLV, failed to activate AM *in vivo*. Water-soluble MDP administered parenterally is cleared rapidly (<1 hr) from the body and excreted in the urine (27). A short exposure of host immune cells to MDP probably is not sufficient to produce appreciable biological activity (Table 2). AM were incubated for 4 hr in free MDP at 50 μg/ml (10 μg/10^5 AM) and then washed and in-

Table 1. Activation of tumoricidal activity in murine AM by injection of free and MLV-encapsulated MDP

Treatment of AM donors*	Radioactivity in live B16 melanoma cells, cpm†		
	4 hr	24 hr	48 hr
None, tumor cells alone	1324 ± 28	1366 ± 23	1115 ± 27
DB saline injection	1401 ± 37	1344 ± 52	1169 ± 63
Free MDP (200 μg)	1398 ± 54	1381 ± 77	1172 ± 40
MLV-MDP (2.5 μg)‡	932 ± 11 (31%)	831 ± 36 (38%)	962 ± 15 (17%)
"Empty" MLV and free MDP (2.5 μg)§	1311 ± 35	1352 ± 23	1146 ± 34

* Three mice per group were injected intravenously with the indicated materials.

† AM were harvested at various times after the intravenous injection and 10^5 were plated into 38-mm^2 culture wells; 1×10^4 [^{125}I]IUdR-labeled B16-BL6 cells were added. Results are mean ± SD of triplicate cultures terminated after 72-hr incubation. Number in parentheses, % cytotoxicity compared with normal (DB saline-injected) AM; $P < 0.01$.

‡ MLV (10 μmol of total phospholipids) containing 2.5 μg of MDP per mouse.

§ MLV (10 μmol of total phospholipids) containing DB saline.

cubated for an additional 20 hr in CME medium prior to assay of their cytotoxicity. This procedure did not render AM tumoricidal. At least 24 hr of incubation of AM with free MDP was required for activation (33% cytotoxicity, $P < 0.01$). Empty MLV plus 0.05 μg of free MDP did not activate AM. In contrast, the addition of MLV (100 nmol of phospholipids) containing 0.05 μg of MDP activated AM even after 4 hr of incubation (45%, $P < 0.01$) and the greatest level of AM activation was observed after 24 hr of incubation (60%, $p < 0.001$).

It is important to note that the amount of MDP delivered to AM in MLV (0.05 μg/10^5 AM) was 1000 times less than that available to mouse AM in wells containing free MDP (50 μg/10^5 AM). Clearly, the internalization of MDP into AM by MLV allows for efficient and rapid activation of macrophages to the tumoricidal state.

Maintenance of the Tumoricidal State *in Vitro*. AM were incubated *in vitro* for 4 hr with free MDP (10 μg/10^5 AM), MLV-encapsulated MDP (0.2 μg/10^5 AM), or MLV containing DB saline plus free MDP (2 μg/10^5 AM). After this 4-hr incubation, the cultures were washed and re-fed with CME medium. Twenty, 44, 68, or 92 hr later, radiolabeled tumor cells were added to the monolayers for the cytotoxicity assays. Only AM treated with MLV-encapsulated MDP were rendered tumoricidal and remained so up to 3 days after incubation with MDP encapsulated within MLV (Fig. 1). This suggested that, to maintain a continuous state of activation, AM need to interact with MLV/MDP every 3 days. Here, and in all other experiments, MLV containing DB saline (empty) in combination with free MDP even at 10 times the amount captured within MLV (the "out" control) failed to render AM tumoricidal. This clearly demonstrates that the activation of AM by MLV-encapsulated MDP is not caused by MLV-mediated alteration in macrophages that enhanced their responsiveness to MDP to a level at which MDP molecules leaking from MLV could induce activation.

Table 2. Kinetics of murine AM activation by free and MLV-encapsulated MDP

Treatment of AM	AM-mediated cytotoxicity, cpm*	
	4 hr MDP then 20 hr CME medium	24 hr MDP
None, tumor cells alone	2950 ± 94	3000 ± 35
Untreated AM	2711 ± 30	2716 ± 43
Free MDP (50 μg)	2557 ± 36	1796 ± 38 (33%)
MLV-MDP (0.05 μg)†	1505 ± 20 (45%)	1094 ± 55 (60%)
"Empty" MLV and free MDP (0.05 μg)‡	2781 ± 63	2777 ± 16

† AM were harvested at various times after the intravenous injection and 10^5 were plated into 38-mm^2 culture wells; 1×10^4 [^{125}I]IUdR-labeled B16-BL6 cells were added. Results are mean ± SD of triplicate cultures terminated after 72-hr incubation. Number in parentheses, % cytotoxicity compared with normal (DB saline-injected) AM; $P < 0.01$.

† MLV (100 nmol of total phospholipid per 10^5 cells) containing 0.05 μg of MDP.

‡ MLV (100 nmol of total phospholipid per 10^5 cells) containing DB saline.

Regression of Spontaneous Pulmonary Metastases After Intravenous Injection of MLV-Encapsulated MDP. To determine the extent of disease present at the time of first treatment, we necropsied several mice. Spontaneous lymph node and pulmonary metastases were well established at the time treatment commenced— i.e., 4–6 weeks after implantation of tumor cells in the footpad and 3 days after leg amputation. Melanotic metastases were visible in the inguinal and superficial iliac nodes (Fig. 2 *a*, and *b*). Parietal pulmonary metastases were visible with a dissecting microscope. Many of these metastases contained thousands of tumor cells (Fig. 2 *c*, and *d*).

In the first set of experiments, all mice were killed 2 weeks after the last treatment. In mice given DB saline, free MDP, or MLV containing DB saline suspended in DB saline containing free MDP, metastases were large and clearly visible (>1 mm in diameter) (Table 3). The B16-BL6 tumor produced metastases in 24 of 28 control mice with a median of 49 metastases per mouse (range 0–107). Intravenuous injections of free MDP or empty MLV plus 2.5 μg of free MDP did not influence the outcome of metastasis. In contrast, multiple intravenous injections of MLV encapsulated MDP significantly decreased the incidence of metastases. Twenty of 27 mice were free of macroscopic tumor foci. There was also a significant reduction in the median number of pulmonary metastases in those mice treated with MLV-encapsulated MDP compared with the other treatment groups (median number of metastases, 0; range, 0–7; $P < 0.001$).

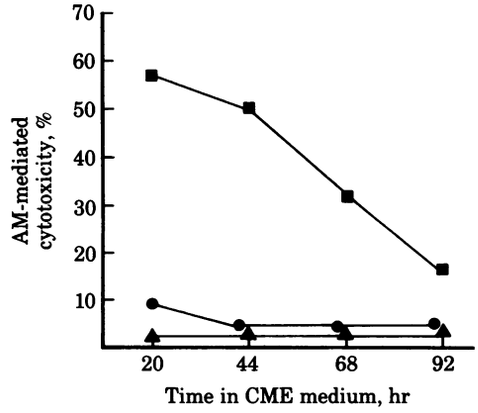

FIG. 1. Duration of AM activation by free and MLV-encapsulated MDP. AM (10^5) were incubated for 4 hr with 10 μg of free MDP (●), MLV containing 0.2 μg of MDP (■), or MLV containing DB saline plus 2 μg of free MDP (▲). Then, the AM were thoroughly washed and re-fed with CME medium. Radiolabeled target cells (1×10^4) were added at various intervals thereafter. Percent cytotoxicity is based on untreated AM (incubated only with CME medium) at corresponding density to target cells ($P < 0.001$). Variation from the mean of triplicate cultures did not exceed 10%.

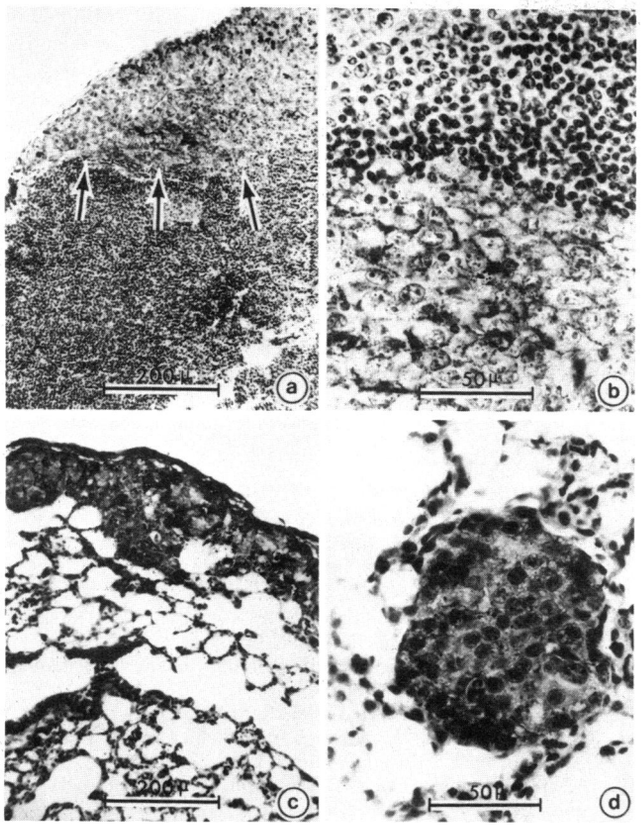

FIG. 2. Spontaneous melanoma metastases observed in lymph nodes (*a* and *b*) and lungs (*c* and *d*) of syngeneic C57BL/6 mice at the start of MLV therapy. Scale bar is 200 μm in *a* and *c* and 50 μm in *b* and *d*.

Survival Studies. The data of the survival experiment are shown in Fig. 3. Each group consisted of 15 or 16 animals. By day 80 of the experiment, nearly all mice in the DB saline, free MDP, or control MLV treatment groups had died. In contrast, 60% of mice (9/16) injected with MLV containing 2.5 μg of MDP per mouse survived 120 days after the last treatment. This period exceeded by a wide margin the time (40–50 days) necessary for as few as 10 surviving tumor cells to kill their hosts (25, 26). On day 190 of the experiment, all the surviving mice were killed, necropsied, and found to be tumor free.

Table 3. Treatment of spontaneous B16-BL6 metastases in C57BL/6 mice by injection of MLV containing MDP

Treatment group	Mice with metastases, no./total	Pulmonary metastases		Mice without visible metastases, %
		Median, no.	Range, no.	
DB saline, control mice	24/28	49	0–107	14
Free MDP (100 μg)	8/10	55	0–94	20
"Empty" MLV and free MDP (2.5 μg)	23/26	68	0–308	12
MLV-encapsulated MDP (2.5 μg)	7/27	0	0–7	74

Viable B16-BL6 cells (25,000) were implanted in the footpad of syngeneic C57BL/6 mice. Limb bearing the tumor (10–12 mm in diameter) was amputated 4 weeks later. Liposome therapy began 3 days after amputation. Each treatment consisted of intravenous injection of MLV (5 μmol of lipid per mouse) twice weekly for 4 weeks. Mice were killed 2 weeks after the last treatment and necropsied. Lung metastases were determined microscopically and confirmed histologically. The incidence of metastasis in the group treated with MLV-encapsulated MDP differed significantly from other groups ($P < 0.001$, χ^2 test). The data represent the combined results of two separate experiments.

DISCUSSION

These studies demonstrate that multiple intravenous injections of MLV-encapsulated MDP, but not free MDP or control MLV preparations, eradicated spontaneous pulmonary and lymph node metastases of a syngeneic melanoma in C57BL/6 mice after resection of the primary tumor. At the start of therapy, metastases in lung and lymph node were grossly visible (Fig. 2). Tumor burden therefore could have exceeded a total of 10^7 cells. Nonetheless, 60% of mice treated with MLV-encapsulated MDP survived at least 120 days after the final treatment [110 days after all control mice died (Fig. 3)]. The tumor burden in these successfully treated mice was probably reduced to <10 viable cells because they survived longer than 40–50 days which is the median life-span of mice implanted with 10 viable B16 cells (25, 26).

The mechanism(s) responsible for regression of established

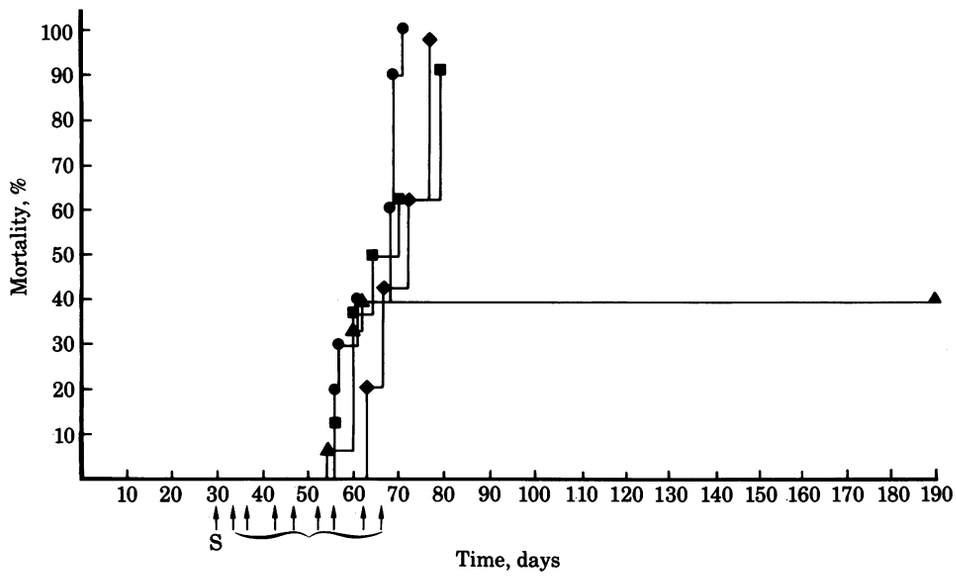

FIG. 3. Survival of mice (15 mice per group) bearing spontaneous lymph node and pulmonary melanoma metastases after multiple intravenous treatments (vertical arrows) with: DB saline (●), 100 μg of free MDP per dose (◆), MLV (5 μmol of phospholipid per mouse) containing DB saline plus 2.5 μg of free MDP (■), and MLV (5 μmol of phospholipid per mouse) containing 2.5 μg of MDP (▲). The difference in survival between mice treated with MLV-encapsulated MDP and all other treatment groups is significant ($P < 0.001$; χ^2 test).

metastases after the systemic administration of MLV containing MDP probably involves the activation of macrophages to become tumoricidal (Table 1). Evidence for the effectiveness of tumoricidal macrophages in controlling cancer metastasis *in vivo* has come from studies in which macrophages activated *in vitro* (28) or *in vivo* (29, 30) were injected intravenously into syngeneic mice bearing experimental or spontaneous metastases. For clinical therapy, however, this approach has serious limitations such as the need to transfer adoptively a large number of autologous or histocompatible macrophages. Because, at least in mice (31, 32), macrophages from tumor-bearing animals can respond to activation stimuli and become tumoricidal, it is preferable to activate them *in situ*. Our present data indicate that the intravenous injection of MLV containing MDP activates AM to become tumoricidal, but the injection of free MDP, even at doses 80 times higher than MLV-encapsulated MDP, does not. This could be due to the fact that water-soluble MDP is cleared from the body within 60 min after parenteral administration and is excreted in the urine (27). Water-soluble MDP administered systemically does not augment cell-mediated immunity (33). To induce hypersensitivity reactions (34), epithelioid granuloma formation (35), adjuvant-induced arthritis (36), and regression of tumors growing subcutaneously (37), MDP needs to be administered in an oil-in-water emulsion.

Retention of MDP after systemic administration can be achieved by capturing the agent with MLV. Our recent work (17) and the data reported here demonstrate that MDP encapsulated within MLV can render mouse or rat AM tumoricidal *in vitro* at concentrations no more than 1/1000th that of free MDP needed to accomplish this task. Moreover, MLV-encapsulated MDP activated AM after 4 hr of incubation *in vitro* or intravenous injection *in vivo*. In order to activate AM with free MDP, a 24-hr incubation period was required. Because water-soluble MDP is cleared from the body in less than 1 hr after parenteral administration (27), this short exposure of macrophages to MDP is not sufficient to produce the activated state. Thus, the intravenous injection of free MDP, even at high doses, does not render macrophages tumoricidal. The encapsulation of MDP within MLV leads to retention of MDP within the macrophage where it could be released over 2–3 days and maintain the tumoricidal state (Fig. 1). This achieves the added advantage of a sustained release of the activating agent.

The ability to activate AM *in situ* with MDP encapsulated with MLV is attractive for several reasons. The repeated systemic administration of MLV is unlikely to lead to the formation of granulomas and elicitation of allergic reactions associated with the systemic administration of some immune adjuvants (7). Once injected into the circulation, MLV like other particulate matter, are removed by cells of the reticuloendothelial system by the process of phagocytosis (21, 23). It is possible to take advantage of this physiological fact to deliver materials encapsulated within MLV to cells of the macrophage-histiocyte series. In these studies we used MLV consisting of PtdSer and PtdCho at a molar ratio of 3:7 for several reasons. First, studies of the distribution of MLV of different size, charge, and chemical composition after intravenous administration demonstrated that increased localization and retention in the lungs were achieved with these MLV (23). Second, extensive toxicity studies in mice and dogs demonstrated that, at the dose used here, these MLV are not toxic (22).

In conclusion, we demonstrate here that the intravenous injection of MLV containing MDP can activate AM to become tumoricidal. Multiple injections of MLV-encapsulated MDP lead to regression of established spontaneous metastases originating from a subcutaneous murine melanoma. The optimal conditions for liposome therapy have not been defined yet. Future studies should determine whether increasing the number of treatments or the dose of MDP in each treatment, or both, could lead to eradication of all metastases or be effective against increased tumor burden. At present, a major reason for the failure of therapy of disseminated cancer is the emergence of metastases resistant to therapy. Regardless of other phenotypic characteristics, at least *in vitro*, tumorigenic cells can be destroyed by activated macrophages. It is therefore possible that activation of macrophages *in vivo* by the systemic administration of MLV containing MDP may be valuable for the treatment of metastases populated by cells that are resistant to the more conventional therapeutic regimens.

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