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A dried preparation of liposomes containing muramyl tripeptide phosphatidylethanolamine as a potent activator of human blood monocytes to the antitumor state

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Summary. Studies were performed on the activation of human blood monocytes to the antitumor state by a dried preparation of multilamellar vesicle (MLV) liposomes in which synthetic muramyl tripeptide phosphatidylethanolamine (MTP-PE) was inserted directly into the liposome membrane. Dried liposomes composed of synthetic phospholipids [phosphatidylcholine (PC) and phosphatidylserine (PS) in a molar ratio of 7 : 3] were prepared by lyophilization. Dried liposome-MTP-PE was found to be superior in several ways to free desmethyl muramyl dipeptide (norMDP) or conventional liposome-MTP-PE, prepared immediately before use. First, dried lipsome-MTP-PE was stable and strongly activated monocytes when stored for over 3 months in a freezer at −20 °C or even in suspension at 4 °C. Second, human monocytes in suspension, as well as in the adherent form, were activated to the tumoricidal state by interaction for at least 4 h with the dried preparation of liposome-MTP-PE. Third, monocytes activated with the dried liposome-MTP-PE or conventionally prepared liposome-MTP-PE maintained their tumoricidal activity for a longer period (4 days) than those activated with free norMDP. These results indicate that the dried preparation of liposome-MTP-PE can be stored for a long time, has a reproducible effect that can be standardized and should be valuable for in situ activation of human monocytes to the tumoricidal state, which is associated with eradication of cancer metastases.

Introduction

Activated macrophages are known to be important in host defense against primary and metastatic tumors [7, 13]. It has been shown previously that the use of liposomes to deliver macrophage activating agents such as lymphokines (MAF) [3, 14, 18, 20, 22] or synthetic muramyl dipeptide (MDP) [18–20, 21, 24, 25] or acyltripeptide and its analogs [26] to monocyte-macrophages resulted in significant induction and potentiation in vitro of the tumoricidal properties of the cells at far lower concentrations and for a longer period than the free forms of these compounds. Moreover, this approach using liposome-encapsulated MAF or MDP was effective in in situ activation of macrophages, which was associated with eradication of established pulmonary metastases [2, 4, 5]. The entrapment of soluble MDP in the aqueous space of liposomes poses problems in terms of its efficient incorporation and subsequent retention [17]. For this reason, the lipophilic analog of MDP (muramyl tripeptide phosphatidylethanolamine, MTP-PE), which could be inserted directly into liposome membrane bilayers, was tested. This MTP-PE entrapped in liposomes was also found to be effective in in situ activation of alveolar macrophages with concomitant destruction of pulmonary metastases in murine systems [2, 5, 8]. However, conventional liposomes containing MTP-PE have to be prepared immediately before use. Moreover, many problems, such as reproducibility, standardization, transportation, and long-term storage of the liposomes have to be overcome before they can be used clinically for treatment of disseminated diseases.

Here, we report that dried preparations of multilamellar vesicle (MLV) liposomes containing MTP-PE, which were prepared by lyophilization of synthetic phosphatidylserine (PS) and phosphatidylcholine (PC) with lipophilic MTP-PE in a vial, are as effective as conventional liposome-MTP-PE in rendering human monocytes tumoricidal. This dried liposomal MTP-PE preparation was stable in a freezer for 5 months, or even in suspension for at least 1 month.

Materials and methods

Target cell lines. Human melanoma A375 cells [26] were a gift from Dr. I. J. Fidler (M. D. Anderson Hospital and Tumor Institute, Houston, Tex.). Monolayer cultures were maintained on plastic in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G, and streptomycin (designated as CRPMI 1640 medium) at 37 °C in a humidified atmosphere of 5% CO_2 in air. For cytotoxicity assays, cultures were used in the exponential growth phase.

Reagents. Soluble hydrophilic norMDP (desmethyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine) (CGP11637) and its lipophilic MDP derivative (MTP-PE; N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2'-dipalmitoyl) phosphatidylethanolamine) (CGP 19835) were gifts from Ciba-Geigy, Co., Basel, Switzerland. None of the reagents contained endotoxins, as shown by *Limulus amoebocyte* lysate assay [25].

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Lipids and conventional preparation of MLV liposomes. Chromatographically pure egg PC and beef brain PS were purchased from Avanti Biochemicals, Birmingham, Ala, and stored under nitrogen in sealed ampules at −70 °C. MLV liposomes containing MTP-PE (5 μg/μmol of lipid) were prepared from a mixture of PC and PS (molar ratio, 5 : 5) by mechanical agitation on a vortex mixer [13, 22, 25]. Lipophilic MTP-PE was dissolved in methanol-chloroform (1 : 2) and added to phospholipids in chloroform. The incorporation and proper orientation of MTP-PE into the phospholipids bilayer membrane was assessed by employing a trace amount of MTP-^{3}H-PE [15]. More than 99% of the radioactivity was associated with vesicles. We used MLV liposomes composed of PS and PC as carrier vehicles for MTP-PE, because at the concentrations used, the liposomes were not toxic to target cells [21]. Liposome preparations were always used within 4 h. On the other hand, dried preparations of MLV liposome-MTP-PE (CGP19835A) were prepared by lyophilization [250 mg of synthetic PC and PS; (molar ratio, 7 : 3) mixed with 1 mg of MTP-PE] in a 15 ml serum vial. The dried liposomes were stored at −20 °C until use. Dried preparations of empty liposomes or liposome-MTP-PE were prepared in Ciba-Geigy Inc., Basel, Switzerland, and sent by air to this laboratory. A liposome suspension was prepared by shaking the lyophilisate with 2.5 ml of phosphate-buffered saline (PBS). The average diameter of the constituted liposomes varied between 2.0 and 3.5 μm, and at least 80% of the liposomes were larger than 1.5 μm, as determined in a Coulter counter TAII with population count accessory (Coulter Electronic Ltd, Luton, England).

Isolation and culture of human peripheral blood monocytes. Leukocyte concentrates were collected from peripheral blood (200 ml), and mononuclear cells were separated from the leukocyte concentrates in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, Md.). Then the monocytes were isolated from the mononuclear cell sample by centrifugal elutriation in a Kubota KR-400 centrifuge with a Hitachi SRR6Y elutriation rotor by a minor modification of the method described previously [27]. Briefly, a fraction containing more than 90% of the total monocyte population was obtained at a speed of 2000 rpm and flow rate of 20 ml/min. More than 95% of these cells were monocytes as determined by nonspecific esterase staining and morphological examination, and more than 97% were viable, as judged by the trypan blue exclusion test. This fraction was washed twice with balanced salt solution, and resuspended in CRPMI 1640 supplemented with 5% FBS, at a concentration of 5×10^5 monocytes/ml. These cells were then plated for 2 h in 96-well Microtest III plates (Falcon Plastics, Oxnard, Calif). After incubation, the nonadherent cells were removed by washing with medium. At this point the purity of the monocytes was >99% judging by their morphology and nonspecific esterase staining.

In vitro activation of monocytes. Monocytes were incubated at 37 °C in medium with or without various amounts of liposome-MTP-PE suspended in CRPMI 1640. After 24 h, the monocytes were washed thoroughly with medium and added to tumor target cells.

Monocyte-mediated cytotoxicity assay in vitro. Monocyte-mediated cytotoxicity was determined by measuring release of radioactivity as described in detail previously [23, 25, 26, 27]. Target cells in the exponential growth phase were incubated for 24 h in CRPMI 1640 with 0.4 μCi/ml ^{125}I-IUdR (sp. act. 5 Ci/mg: Amersham International, Bucks, England). No significant differences were detected in the plating efficiencies of labeled target cells on a plastic surface and on monolayers of monocytes (untreated and treated). The cells were washed twice with warm PBS to remove unbound radiolabel, harvested by brief trypsinization (0.25% Difco trypsin and 0.02% EDTA for 1 min at 37 °C), and suspended in CRPMI 1640; unless otherwise described, 1×10^4 A375 melanoma cells were plated in wells containing monocytes (10^5/well). The cultures were washed 16 h after plating the target cells to remove nonadherent and dead target cells and were refed with fresh CRPMI 1640. Then 56 h later, the monocyte-target cell cultures were washed twice with PBS, and adherent, presumably viable cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed on cotton swabs and placed directly in 12 × 75 mm tubes, and the radioactivity counted. Thus, the amount of radioactivity associated with the DNA of adherent viable cells was used for measuring the degree of monocyte-mediated cytotoxicity (target cell lysis). Preliminary experiments indicated that more than 80% of the radioactivity in the supernatants obtained by washing 72-h cultures of test monocytes and target cell monolayers was associated with material that passed through a 0.45-μm millipore filter.

The percent cytotoxicity mediated by treated human monocytes was calculated as follows:

$$\text{Percentage of specific cytotoxicity} = 100 \times \frac{A - B}{A},$$

where A = cpm in cultures of untreated monocytes and target cells, and B = cpm in cultures of test monocytes and target cells. The statistical significance of differences between test groups was analyzed by Student's two-tailed *t*-test.

Results

Activation of tumoricidal properties in human monocytes by dried liposome-MTP-PE or freshly prepared liposome-MTP-PE

First, human blood monocytes isolated by centrifugal elutriation were treated for 24 h with medium containing different amounts of dried or freshly prepared liposome-MTP-PE (5 μg/μmol), or dried empty liposomes. Results of representative experiments are shown in Fig. 1. Untreated human monocytes were not cytotoxic to allogeneic A375 melanoma cells, and their incubation in vitro with dried liposomal MTP-PE, like freshly prepared liposome-MTP-PE at concentrations of 10 nmoles to 200 nmoles resulted in significant activation of the tumoricidal activity of the monocytes. In contrast, dried empty liposomes at concentrations of less than 100 nmoles/10^5 cells did not induce tumoricidal monocytes, although 200 nmoles/well was somewhat effective. In parallel experiments, we varied the monocyte/target cell ratio by plating a constant number of target cells per well. The cytotoxicity mediated by monocytes treated with dried or conventionally prepared liposome-MTP-PE was increased by introduction of more

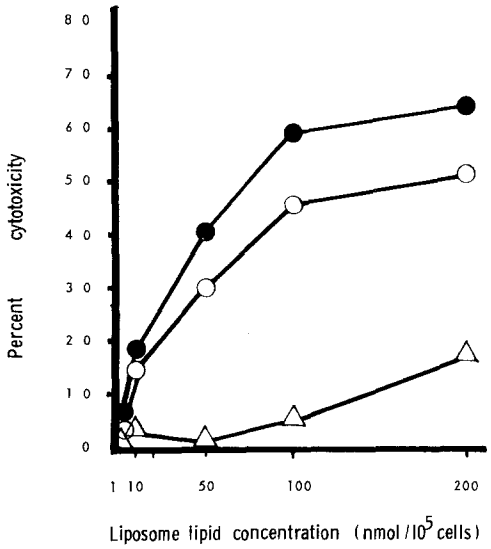

Fig. 1. Activation of human blood monocytes by dried or conventionally prepared liposome-MTP-PE. Monocyte monolayers were treated for 24 h with the indicated amounts of dried (●) or conventional (○) preparations of liposome-MTP-PE, or empty dried preparations of liposomes (△) before addition of labeled A375 melanoma cells. Assays were terminated 72 h later. The percentage cytotoxicity of treated monocytes was determined with reference to results for untreated monocytes and tumor cells as described in *Materials and methods.* SD < 10% of the mean value. Data are representative one of four separate experiments

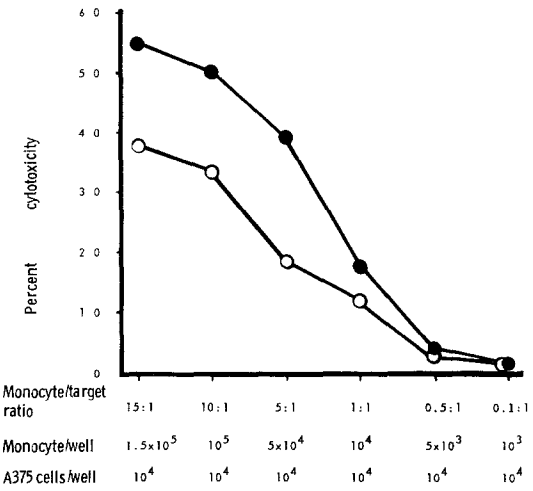

Fig. 2. Effect of the ratio of monocytes to target cells on the level of monocyte-mediated cytotoxicity. Different numbers of monocytes in monolayers were treated for 24 h with medium containing 100 nmoles/well of dried (●) or conventional (○) preparations of liposome-MTP-PE, and washed thoroughly before addition of 10^4 labeled A375 melanoma cells. Assays were terminated 72 h later. Percent cytotoxicity by treated monocytes was calculated as in Fig. 1

monocytes into the well. Significant and reproducible levels of cytotoxicity were obtained by incubating at least 10^4 monocytes with 10^4 target A375 melanoma cells (Fig. 2).

Next, we examined whether monocytes in suspension can be rendered tumoricidal by interaction with dried liposome-MTP-PE, since for therapeutic purposes liposome-MTP-PE should be administered i. v. to be phagocytized by monocytes circulating in the blood. Human monocyte suspensions in polypropylene tubes were incubated in medium with different amounts of liposome-MTP-PE. Data are given in Table 1. Human monocytes in suspension, like adherent monocyte monolayers, were acvated to the tumoricidal state by interaction for 24 h with dried liposome-MTP-PE. Moreover, monocytes in suspension when treated for 4 h with dried liposome-MTP-PE and then incubated in medium alone for 20 h caused significant lysis of A375 melanoma cells.

Table 1. Activation of human monocytes in adherent or suspension culture by liposomes containing MTP-PE

Monocyte treatment[a]	Adherent monocytes treated for 24 h	Monocyte suspension treated	
		for 4 h	for 24 h
A375 cells alone	1645 ± 63[b]		
Medium	1664 ± 101	1671 ± 55	1787 ± 19
Fresh liposome-MTP-PE			
50 nmoles	1027 ± 60 (38)[c]	1458 ± 42 (13)	1247 ± 98 (30)[c]
100 nmoles	1048 ± 11 (37)[c]	1328 ± 107 (21)[c]	1147 ± 90 (36)[c]
Dried liposome-MTP-PE			
50 nmoles	1052 ± 19 (37)[c]	1208 ± 40 (28)[c]	1238 ± 45 (30)[c]
100 nmoles	864 ± 56 (48)[c]	1103 ± 95 (34)[c]	867 ± 77 (51)[c]
Dried empty liposomes			
50 nmoles	1651 ± 38	1591 ± 71	1726 ± 72
100 nmoles	1680 ± 7	1706 ± 82	1649 ± 62

[a] Human monocytes (10^5) in suspension were treated with the indicated agents for 4 h or 24 h, and then plated in wells of Microtest III plates, washed and incubated with fresh CRPMI 1640 for 20 h or 0 h, respectively, before addition of labeled A375 melanoma cells. Assays were terminated 72 h later

[b] Cpm ± SD for triplicate cultures. Data are representative of four separate experiments

[c] Percent cytotoxicity mediated by treated monocytes calculated based on the values for untreated monocytes ($p < 0.05$)

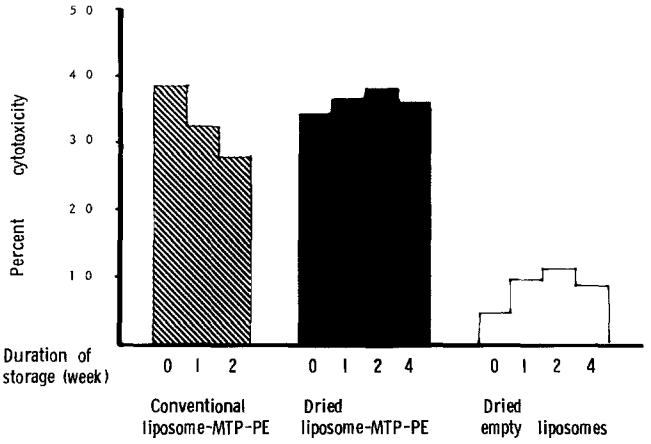

Fig. 3. Stability of dried liposome-MTP-PE in suspension. Dried preparations of liposomes with or without MTP-PE were reconstituted with PBS, and stored at 4 °C. At the same time, conventional liposome-MTP-PE was prepared and stored similarly in suspension. At the indicated times, the activities of these liposome-MTP-PE preparations in monocyte-mediated cytotoxicity assay were examined as described in *Materials and methods.* SD < 10% of the mean value. Data are representative one of three separate experiments

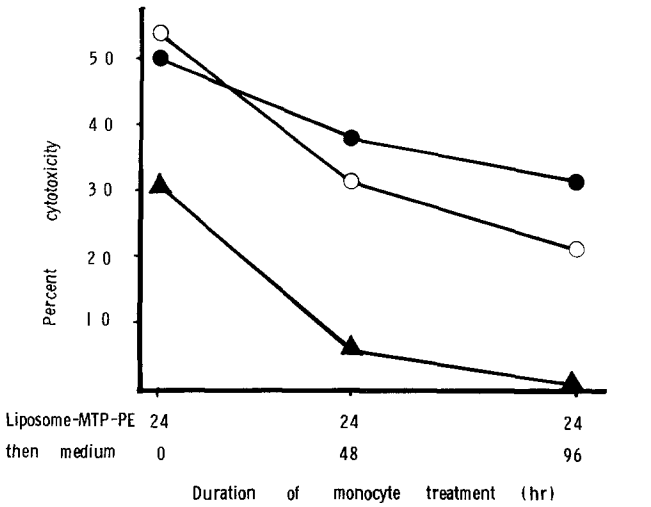

Fig. 4. Maintenance of tumoricidal activity of monocytes activated for 24 h with dried or conventional liposome-MTP-PE. Freshly isolated monocytes were incubated in medium containing 100 nmol/10^5 cells of dried (●) or conventional (○) liposome-MTP-PE, or 50 μg/ml of norMDP (△). After treatment, the monocyte monolayers were washed and incubated in fresh medium. At the indicated times, 10^4 labeled A375 melanoma cells were added to the monocyte cultures and incubations were terminated 72 h later. Percent cytotoxicity by treated monocytes was determined as for Fig. 1. Data are representative one of four separate experiments. SD < 10% of the mean value

Effects of long-term storage of dried liposome-MTP-PE in a freezer or in suspension on monocyte activation

Dried liposome-MTP-PE was prepared in Ciba-Geigy Inc., Basel, Switzerland, and sent by air to Japan. Then, the preparations were stored in a freezer at −20 °C until use. Some vials containing liposome-MTP-PE were thawed, suspended in PBS, and again stored at 4 °C before testing. In preliminary experiments, dried preparations stored for up to 5 months were all effective in human monocyte activation, whereas control empty liposomes were not effective (data not shown). Figure 3 shows that dried preparations of liposome-MTP-PE (100 nmoles/10^5 monocytes) that had been stored in suspension at 4 °C for 4 weeks, were as effective in activation of monocytes as fresh liposome-MTP-PE suspensions, indicating the stability of dried liposome-MTP-PE for monocyte activation. Moreover, there was no significant difference in monocyte activation between conventional and dried preparations of liposome-MTP-PE stored in suspension for 4 weeks.

Maintenance of tumoricidal activity of human monocytes activated with dried liposome-MTP-PE

The duration of activation of human monocytes after in vitro treatment with medium containing dried liposome-MTP-PE or freshly prepared liposome-MTP-PE is shown in Fig. 4. Human monocytes were treated for 24 h with 100 nmol of MLV liposome-MTP-PE. Then the cultures were washed, refed with fresh medium, and radiolabeled target A375 cells were added to the monocyte monolayers for cytotoxicity assay 0, 48, and 96 h after monocyte-liposome-MTP-PE interaction. Monocytes treated with dried or freshly prepared liposome-MTP-PE were highly cytotoxic immediately after activation and remained tumoricidal up to the end of the assay at 96 h. Thus, human monocytes that phagocytosed an optimal amount of MLV liposome-MTP-PE maintained higher cytotoxicity for a longer period than monocytes treated with soluble MDP.

Discussion

The present study demonstrated that human monocytes can be rendered tumoricidal by interaction in vitro with a dried preparation of liposomes in which MTP-PE, a lipophilic analog of MDP, is inserted into membrane bilayers, and that the strong activity of dried liposomal MTP-PE to activate human monocytes was maintained on storage of the preparation for a long time in a freezer at −20 °C or even in suspension at 4 °C.

Many biological response modifiers (BRM), such as MAF [3, 10, 14, 18, 20, 22], C-reactive protein (CRP) [1], gamma interferon [15, 16], acyltripeptide and its analogs [26] and MDP and its analogs [9, 11, 18–20, 21, 24, 25] have been encapsulated within liposomes for effective activation of human or murine monocyte-macrophages to the tumoricidal state in vitro. We and others have shown that several BRMs, such as MDP and its analogs [5, 17], MAF [6] and CRP [1], when encapsulated in liposomes, are effective in eradication of pulmonary metastases on their systemic administration. Nevertheless, encapsulation of hydrophilic BRM (MAF or MDP) into the aqueous space of MLV for effective induction in vitro and in situ of tumor cytotoxicity mediated by monocyte-macrophages was found to be associated with poor retention of these hydrophilic BRM in liposomes during longterm storage [17]. For this reason, the lipophilic analog of MDP was inserted directly into membrane bilayers, and we previously showed that it was equally effective for in vitro and in situ activation of macrophages [5, 23, 25]. The present study using dried preparations of MLV liposome-MTP-PE supported and confirmed our previous findings [23, 25] that MLV liposome-MTP-PE prepared freshly before use caused marked induction of monocyte-mediated tumor cytotoxicity.

In the present studies, we examined the effect and stability of a dried preparation of liposome-MTP-PE composed of synthetic PC and PS in a molar ratio of 7 to 3, on the activation of human monocyte-mediated cytotoxicity. We used liposomes of this composition, since previous studies [2, 21] demonstrated that for maximal activation in vitro and in situ of murine or human alveolar macrophages by MDP, the optimal composition of MLV liposomes was PC and PS in a molar ratio of 7 to 3. The size distribution of dried liposome-MTP-PE was almost the same as that of freshly prepared MLV liposomes (ratio of PC to PS, 7:3) reported previously [2]. Moreover, it is noteworthy that this dried preparation of liposome-MTP-PE was sent to Japan by air from Ciba-Geigy Inc., Basel, Switzerland without loss of macrophage activating potential. This fact, and the above findings indicate that the dried preparation of MLV liposome-MTP-PE composed of synthetic materials can be used as a reproducible and standardized macrophage activating agent, which is now universally available for therapeutic clinical trials.

The present studies on the kinetics of monocyte activation by dried liposome-MTP-PE indicated that maximal activation of human monocytes to the tumoricidal state was observed 24 h after the initial treatment, and the tumoricidal activity was maintained for 72 h on incubation in the medium. This observation was in accordance with previous findings on murine and human macrophage activation by conventionally prepared liposomal MDP and MTP-PE [4, 21, 25], suggesting that liposomes may have a depot-like function in allowing the interaction between monocytes and MTP-PE inserted in liposome membrane bilayers, although the exact mechanism by which lysosomal enzymes disrupt and degrade liposome-MTP-PE remains to be studied.

Phillips et al. [12] recently demonstrated in murine systems that freeze-dried liposomes containing a lipohilic MDP derivative that allowed the preparation and long-term storage of reproducible liposome formulations, was a potent inducer of murine macrophage-mediated cytotoxicity in vitro and in situ, and had antitumor activity in vivo. This finding was confirmed and extended by the present observations that dried preparations of liposomes containing MTP-PE were effective in activating human monocytes to the tumoricidal state in vitro.

The present observation that the tumoricidal activity of blood monocytes can be induced by direct interaction of the cells with dried liposomes containing MTP-PE is very important from an immunotherapeutic point of view, since systemic administration of these liposomal agents should lead to their interaction with circulating monocytes, and then to their phagocytosis by the monocytes. In fact, we showed previously that human monocytes phagocytized MLV liposomes in a dose-dependent manner [23]. When monocytes in circulation have phagocytized liposome-MTP-PE, they probably migrate out of the blood vessels into the sites of growing tumors, and as monocyte-macrophages with antitumor activity they eradicate metastatic tumors. This idea is supported by the finding of Key et al. [8] that macrophages isolated from lung tumor colonies of mice that had been treated i. v. with liposome-MTP-PE showed tumoricidal activity, since MLV liposomes themselves cannot pass through the vessel wall directly because they are too large. Thus, these findings indicate that dried preparations of liposome-MTP-PE (Ciba-Geigy) should be effective for in situ activation of human blood monocytes to eradicate cancer metastases.

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