Activation of Tumoricidal Properties in Monocytes from Cancer Patients following Intravenous Administration of Liposomes Containing Muramyl Tripeptide Phosphatidylethanolamine

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ABSTRACT

This study examined the antitumor properties of blood monocytes isolated from patients undergoing a phase I trial with liposomes containing muramyl tripeptide phosphatidylethanolamine (L-MTP-PE). Peripheral blood monocytes were isolated from 28 patients receiving twice weekly i.v. injections of escalating doses of L-MTP-PE. Monocytes were harvested before therapy and at various times during the 9-week treatment period. Activation of monocyte-mediated tumoricidal activity was found in 24 of the 28 patients at some time during treatment. Whereas the maximum tolerated dose of L-MTP-PE was 4-6 mg/m², the optimal biological dose in terms of macrophage activation was 0.5-2.0 mg/m². The spontaneous secretion of interleukin 1 from monocytes isolated pre- and postinfusion was monitored in two patients. In both patients interleukin 1 secretion correlated with the cytotoxic activity of the monocytes. We conclude that the systemic administration of L-MTP-PE can render the blood monocytes of cancer patients tumor cytotoxic. Since L-MTP-PE is an immunomodulator devoid of direct antiproliferative effects on tumor cells, the data suggest that future clinical trials be conducted using the optimal biological dose rather than the maximum tolerated dose.

INTRODUCTION

MDP is the minimal structural unit of mycobacteria with immunopotentiating activity (1, 2). Although MDP readily activates the tumoricidal properties of both human (3) and rodent (4) macrophages in vitro, it is not suitable for therapeutic use in vivo because it is cleared from the circulation within 60 min of parenteral administration (5, 6), an exposure time that is not sufficient to render macrophages tumoricidal (7-10).

Encapsulating the MDP in multilamellar vesicles, or liposomes, can overcome this problem by delivering the activating agent in a carrier vehicle that is rapidly and efficiently phagocytized by monocytes and macrophages. Maximal uptake of the liposomes is attained after 1 h (10, 11) which provides sufficient time for the activating agents to be introduced to the effector cells, producing tumoricidal properties several hours thereafter. Indeed, liposome-encapsulated MDP was very efficient in rendering rodent macrophages tumoricidal in vivo, and its systemic administration eradicated established pulmonary and lymph node metastases (7). Unfortunately, water-soluble MDP leaks out of the liposomes at a rate of 10-20%/h depending on the phospholipid composition (4).

The synthetic lipophilic analogue of MDP, MTP-PE, can be incorporated directly into the lipid bilayers of multilamellar liposomes with high efficiency and without measurable leakage (2, 10, 12). L-MTP-PE are more efficient than liposomes containing MDP at activating the tumoricidal properties of monocytes and macrophages in vitro (2-4, 13). Moreover, in vivo administration of L-MTP-PE produces in situ activation of tumoricidal properties in macrophages (14); the regression of established lymph node, lung, and liver metastases in mice (7, 10); a delay in the development of autochthonous UV-B-induced skin cancers in mice (15); inhibition of the development of metastatic spread (16); and an enhancement of the host’s defenses against viral (17, 18) and fungal (19) diseases. Most important, repeated i.v. administration of L-MTP-PE has been effective in the therapy of dogs with metastatic osteosarcoma (20).

A stable, pharmaceutical grade preparation of L-MTP-PE (21) has been produced by Ciba-Geigy, Basel, Switzerland, for clinical use in humans. Because the systemic activation of macrophages is thought to be a major mechanism by which L-MTP-PE produces antitumor effects (10, 22) we wished to determine the extent of in situ activation of blood monocytes in patients undergoing phase I study.

MATERIALS AND METHODS

Reagents and Drugs. RPMI 1640, HBSS, FBS, human AB serum, and Eagle’s complete minimal essential medium (M. A. Bioproducts, Walkersville, MD) were used. All reagents were free of detectable endotoxins as determined by the Limulus amebocyte lysate assay (sensitivity limit, 0.025 ng/ml). Freeze-dried L-MTP-PE (CGP 19835A) was supplied by Ciba-Geigy Ltd., (Summit, NJ). Free MTP-PE is a colorless, odorless powder that is stable for >2 y at -20°C. It is unstable in aqueous solutions under pH 4 or over pH 7. MTP-PE was incorporated into phospholipid liposomes made of synthetic phosphatidyserine and phosphatidylcholine in a 3:7 molar ratio. The MTP-PE:phospholipid ratio was 1:250 (mg/mg). L-MTP-PE was prepared from the lyophilized product by hydrating the lipids with 3 ml of normal saline and then vortexing for 5 min. The preparation was then diluted in 50 ml normal saline and administered i.v. over 1 h using an infusion pump.

Patients. Twenty-eight patients with metastatic cancer refractory to standard therapy were entered in a phase I trial with L-MTP-PE (CGP 19835A). Patients had an estimated performance status of 0-2 using Zubrod’s criteria and an estimated life span ≥12 weeks. The patients gave written informed consent to participate in the study according to our institutional policy. Patient characteristics are summarized in Table 1. Patients had not received any antitumor therapy for a minimum of 2 weeks before entering this study. L-MTP-PE was infused i.v. over 1 h twice weekly for a total duration of 9 weeks. The first three patients in the study received L-MTP-PE at a dose of 0.05 mg/m². After 3 weeks of twice weekly infusions, the patients were escalated to the next dose level (0.1 mg/m²) for 3 weeks and subsequently to a third and final level of 0.25 mg/m² twice weekly for 3 weeks. If no major toxicity was observed, an additional three patients were entered at a dose one level higher than the initial starting level for the previous group of

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The abbreviations used are: MDP, muramyl dipeptide; MTP-PE, N-acetylglucosamine-1-α-mannosaminyl-2-α-fucosyl-3-phosphoryl)ethylenemaltolite; HBSS, Hanks’ balanced salt solution; FBS, fetal bovine serum; L-MTP-PE, liposomes containing MTP-PE; MNL, mononuclear leukocytes; rIFN-γ, human recombinant γ-interferon; IL-1, interleukin 1; TNF, tumor necrosis factor α; rGM-CSF, human recombinant granulocyte/macrophage colony-stimulating factor; MTA, monocyte tumoricidal activity.

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cell suspension was adjusted to contain 1 x 10^6 monocytes/ml. Into containing 5% human AB serum. The percentage of monocytes in the carried out immediately before administration of L-MTP-PE and at 1, viruses.

plastic in medium supplemented with 5% FBS, sodium pyruvate, non-described previously (3, 11). Monolayer cultures were maintained on

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mens were obtained from 10 normal donors weekly for 3 weeks. Initial deter

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doses of L-MTP-PE thus administered were 0.05, 0.10, 0.25, 0.50, 1.0,

patients. This dose was escalated every 3 weeks again for a total duration of 9 weeks (Table 2). There were no "rest" periods between doses. The doses of L-MTP-PE thus administered were 0.05, 0.10, 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 9.0, and 12.0 mg/m^2 (23).

Sampling. Peripheral blood monocytes were isolated from 20 ml of heparinized blood and assayed for tumoricidal activity. Initial determinations of monocyte activity in peripheral blood monocytes were carried out immediately before administration of L-MTP-PE and at 1, 24, 72, and 96 h postadministration. For control studies, blood specimens were obtained from 10 normal donors weekly for 3 weeks.

Isolation of Human Monocytes. MNL were isolated from peripheral blood on lymphocyte separation medium (Organon Teknika, Durham, NC), washed twice in HBSS, and then suspended in RPMI 1640 containing 5% human AB serum. The percentage of monocytes in the MNL layer was assessed by morphology and peroxidase stain, and the cell suspension was adjusted to contain 1 x 10^6 monocytes/ml. Into each well of a 96-well flat-bottomed Microtest II plate (Falcon Plastics, Oxnard, CA), 2 x 10^5 monocytes were added and allowed to adhere for 1 h at 37°C. Nonadherent cells were removed by 3 washes with HBSS. The purity of the adherent monocyte monolayers was >97% as assessed by India ink ingestion, morphology, and peroxidase staining. Purified monocytes were then incubated at 37°C for 18-24 h with 0.2 ml control medium, medium containing rIFN-γ and MDP, or medium containing lipopolysaccharide. After this incubation period the adherent monocyte cultures were washed with HBSS, and [125I]iododeoxyuridine-labeled target cells were added as described below.

Target Cell Cultures. The cultured cell line A375, derived from a human melanoma (23), was used as human tumor target cells as described previously (5, 11). Monolayer cultures were maintained on plastic in medium supplemented with 5% FBS, sodium pyruvate, non-essential amino acids, twice concentrated vitamin solution, and L-glutamine (M. A. Bioproducts) in a humidified atmosphere of 5% CO2 in air. All cultures were free of Mycoplasma and pathogenic murine viruses.

Assay of Monocyte/Macrophage-mediated Cytotoxicity against Tumor Target Cells. Monocyte-mediated tumor cytotoxicity was assessed by a modification of a radioactive release assay as described previously (3, 11). Target cells in the exponential growth phase were incubated for 24 h in supplemented medium containing 0.3 µmol [125I]iododeoxyuridine (New England Nuclear, Boston, MA). The A375 cells were washed twice to remove unbound radiolabel and harvested by a 1-min trypsinization with 0.25% trypsin (Difco, Detroit, MI) and 0.02% EDTA. The labeled cells were washed with medium and resuspended in the supplemented medium, and 1 x 10^4 cells were plated into the culture wells with the macrophage cultures to obtain an initial target:effector cell ratio of 1:20. Radiolabeled target cells plated alone provided an additional control group. After 24 h, the cultures were washed to remove the nonadherent target cells, refed with fresh medium, and then cultured for an additional 48 h. The cultures were then washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter. The percentage of tumor cells undergoing monocyte-mediated cytolysis was calculated by two different formulas. In the first, we calculated the cytolysis mediated by monocytes collected from cancer patients without further in vitro treatment as

\[
\text{Cytotoxicity} \% = \frac{A - B}{A} \times 100
\]

where A is the cpm of target cells cultured alone and B is the cpm of target cells cultured with monocytes. We determined previously that natural killer cell activity does not contribute to the cytotoxicity assay (24) and that this assay measures lysis rather than target cell detachment (11).

For assays measuring the in vitro activation of monocytes by lipopolysaccharide, L-MTP-PE, or a combination of rIFN-γ and MDP, the generated cytotoxicity was calculated as

\[
\text{Generated cytotoxicity} \% = \frac{A - B}{A} \times 100
\]

where A is the cpm of target cells cultured with untreated monocytes, and B is the cpm of target cells cultured with in vitro-activated monocytes.

Statistical Analysis. The results were analyzed for their statistical significance by Student's t test (two-tailed).

Supernatants for IL-1 and TNF Assay. Patient monocytes were separately incubated for 24 h with RPMI 1640 containing 5% AB serum or the indicated immunostimulants. The culture supernatants were harvested, centrifuged at 225 x g for 5 min to remove residual cells and cellular debris, and stored at -20°C.

IL-1 Assay. The murine helper T-cell clone D10.G4.1 provided by Dr. C. Janeway (Yale University, New Haven, CT) was used to assay IL-1 activity. Every 3 weeks, the cell line was carried in interleukin 2 supplemented Click's medium (25) passed on C57BL/6 spleen cells every 3 weeks. The C57BL/6 cells provided both the alloantigen H-2b recognized by the D10.G4.1 clone and the feeder effect. The D10.G4.1 murine cell line assay for IL-1 was performed basically as described (25) with the following modifications. D10.G4.1 cells were cultured in bulk quantities for 14 to 16 days following exposure to H-2b antigen-presenting spleen cells (C57BL/6) and frozen at 2 x 10^6 cells/aliquot in medium containing 20% FBS and 10% dimethyl sulfoxide. On the day of the assay, cells were quickly thawed, washed twice in medium, and seeded at 1 x 10^6 cells/well in medium containing 2.5 µg/ml concanavalin A (Sigma, St. Louis, MO), together with serial dilutions of the sample, in 96-well microtiter plates. Following 48 h in culture, 0.2 µCi of [3H]thymidine was added to each well, and the plates were harvested as described previously (26, 27). The use of frozen D10.G4.1 cells did not affect the sensitivity of the assay or the magnitude of the response but rather provided a more consistent, reproducible assay (27).

RESULTS

Effect of i.v. Infusions of L-MTP-PE on WBC and Percentage of Monocytes. The repeated i.v. administrations of L-MTP-PE did not significantly increase the number of circulating monocytes, which contrasted with our experience with rGM-CSF

<table>
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<th>Group</th>
<th>Dosage (mg/m²)</th>
<th>No. of patients</th>
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<tr>
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Table 2 Protocol Treatment Schema

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<td>19/9</td>
<td>50</td>
<td>18-71</td>
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(28). In the present study we obtained an average of $<4.0 \times 10^5$ monocytes/ml of whole blood from all 28 patients, a value that corresponds to that previously reported for normal individuals (28). This number of monocytes remained constant throughout the study.

Cytotoxicity of Monocytes from Patients Receiving L-MTP-PE. Because L-MTP-PE has previously been reported to activate the tumoricidal properties of normal human monocytes under in vitro conditions (3), we evaluated the antitumor response of blood monocytes from patients receiving systemic administrations of L-MTP-PE. Blood samples were obtained before therapy and at 1, 24, 72, and 96 h postadministration. These samples were collected at the first administration of L-MTP-PE and subsequent to each dose escalation. Monocytes harvested from all patients before therapy could be activated in vitro to kill tumor cells by incubation with lipopolysaccharide, a combination of rIFN-γ and MDP, and L-MTP-PE. These data show that the patients' monocytes were not defective in this response.

The phase I protocol using L-MTP-PE required escalation of the drug dosage twice over the 9-week treatment period. Therefore, all patients received more than one dose level of the drug. Although we demonstrated activation of monocyte antitumor cytotoxic properties in 24 of the 28 patients at some time during the treatment period, the intrapatient drug escalations ruled out a simple analysis of which dose, if any, produced the optimal biological effects. We therefore analyzed the data in several ways.

Patients were divided into two groups. Group A (20 of 28 patients) demonstrated an initial monocyte-mediated cytotoxic activity $<35\%$ prior to administration of L-MTP-PE (range, 0–34%; mean percentage of cytotoxicity $\pm$ SEM = 17 $\pm$ 2). The mean monocyte-mediated cytotoxicity value for normal individuals used in parallel studies was 11 $\pm$ 2 (range, 0–33%) and did not differ significantly from that of this patient population ($P > 0.05$). The monocyte-mediated cytotoxicity value of $<35\%$ was chosen because it is 2 SD above the normal mean. In group B (8 of 28 patients), the pretherapy values were $\geq 35\%$. The reason for the initial elevated monocyte-mediated antitumor activity in these patients is unclear. The 4 patients whose monocytes did not exhibit increased antitumor cytotoxic activity with L-MTP-PE treatment were in group B.

Results from the monocyte-mediated cytotoxicity assays are shown in Figs. 1 and 2. In Fig. 1A, patients from group A (baseline, $<35\%$) were subdivided according to the average dose of L-MTP-PE received. The results are expressed as change in percentage of MTA. Significant change in MTA was demonstrated in those patients who had received an average of 0.55–2.0 mg/m$^2$ of L-MTP-PE twice weekly. In Fig. 2A the patients from group A (baseline, $<35\%$) were subdivided according to the starting dose of L-MTP-PE. Once again, the results are expressed as change in percentage of MTA. Each patient underwent 2 dose escalations during the treatment period. Significant change in the MTA was found in the group of patients whose starting dose of L-MTP-PE was 0.5, 1.0, and 1.5 mg/m$^2$ (see legend to Fig. 2A).

If the data were analyzed by single dose of L-MTP-PE given rather than by individual patient (i.e., every patient that received 1.5 mg/m$^2$ would be included in the analysis of the monocyte-mediated cytotoxicity produced by 1.5 mg/m$^2$ whether the patients received an initial dose of 1.5 mg/m$^2$ and then were escalated to a higher dose or received an initial dose of less than 1.5 mg/m$^2$ and then were escalated to the 1.5-mg/m$^2$ level), patients who received 0.5–2.0 mg/m$^2$ in a single dose demonstrated a significant increase in MTA at 72 h after infusion ($P < 0.006$, data not shown). We therefore concluded that, in patients whose baseline MTA was $<35\%$, an i.v. infusion of 0.5–2.0 mg/m$^2$ L-MTP-PE produced the optimal activation of blood monocyte-mediated cytotoxicity.

The data obtained from patients in group B (baseline, $\geq 35\%$) were analyzed in a similar fashion. As shown in Figs. 1B and 2B, significant decreases in MTA were observed in patients who received i.v. infusions of 0.5–6.0 mg/m$^2$ L-MTP-PE calculated as either an average or starting dose.

The antitumor activity of monocytes harvested during therapy from all 28 patients (groups A and B) at every dose level could be enhanced by in vitro incubation with lipopolysaccharide, rIFN-γ and MDP, or L-MTP-PE. The monocytes were incubated for 24 h with medium and then assayed for cytotoxicity against $[^{3}H]$-iododeoxyuridine-labeled A375 tumor target cells in a 72-h assay. Results are expressed as change in percentage of cytotoxicity (post dosing-pretherapy). Patients are divided into two groups: A, cytotoxicity baseline value of $<35\%$ (20 of 28 patients); B, cytotoxicity baseline value of $\geq 35\%$ (8 of 28 patients). The groups were subdivided according to the average dose of L-MTP-PE each patient received. Statistical significance was determined by the Student's t test (two tailed) (*, $P < 0.05$; **, $P < 0.005$). Bars, SE.

**Fig. 1.** Effect of L-MTP-PE administration on monocyte tumoricidal activity. Peripheral blood monocytes were isolated from patients prior to L-MTP-PE therapy and then at 1, 24, 72, and 96 h postadministration. Monocytes were incubated for 24 h with medium and then assayed for cytotoxicity against $[^{3}H]$-iododeoxyuridine-labeled A375 tumor target cells in a 72-h assay. Results are expressed as change in percentage of cytotoxicity (post dosing-pretherapy). Patients are divided into two groups: A, cytotoxicity baseline value of $<35\%$ (20 of 28 patients); B, cytotoxicity baseline value of $\geq 35\%$ (8 of 28 patients). The groups were subdivided according to the average dose of L-MTP-PE each patient received. Statistical significance was determined by the Student's t test (two tailed) (*, $P < 0.05$; **, $P < 0.005$). Bars, SE.
accompained by the secretion of IL-1 which decreased following the administration of L-MTP-PE. The cytotoxic activity of these monocytes also decreased following L-MTP-PE infusion. By contrast, the monocytes of patient 1 did not exhibit cytotoxicity and did not produce IL-1 before treatment. However, after infusion with L-MTP-PE both antitumor cytotoxicity and IL-1 secretion were found.

The monocytes from two additional patients exhibiting high baseline cytotoxic values (≥35%) also secreted IL-1 prior to therapy. They received starting L-MTP-PE doses of 1.5 and 2.0 mg/m2. Both IL-1 secretion and antitumor cytotoxic activity decreased in these patients as well following infusion with L-MTP-PE (data not shown).

**DISCUSSION**

The present study evaluated the antitumor response of blood monocytes from cancer patients treated systemically with L-MTP-PE during a phase I trial. The initial MNL count was measured. The percentage of monocytes in the MNL layer and the yield of monocytes/ml of whole blood obtained from the patients were monitored throughout the 9-week L-MTP-PE administration period. No significant increase in monocyte yield was found. This result contrasted with the dose-dependent increase observed with rGM-CSF infusion (28). We have previously determined that monocyte yields and cytotoxic activity remain stable in normal healthy donors (28). Like monocytes from normal donors (3, 11) and from patients with a variety of malignancies (28, 29), peripheral blood monocytes isolated from all 28 patients in this study could be activated in vitro to lyse allogeneic tumor cells in a 72-h cytotoxicity assay. Thus, we found no inherent “defect” in the monocytes of the patients in the phase I trial.

Optimal systemic *in situ* activation of human blood monocytes by parenteral administration of biological agents does not require the administration of the agents at the maximal tolerated dose. For example, the i.v. or i.m. administration of rIFN-γ generated tumoricidal blood monocytes *in vivo* in a dose-related fashion (29). Specifically, 0.25 mg/m2/day i.v. or 0.25–0.5 mg/m2/day i.m. was the optimal biological dose measured by the level of monocyte-mediated tumor cell killing. Doses of rIFN-γ below 0.25 mg/m2/day were not effective, whereas 1 mg/m2/day doses produced toxicity to monocytes and monocytes were refractory even to *in vitro* activators (29). The systemic administration of rGM-CSF alone did not generate cytotoxic monocytes in any dose level (28), but cytotoxicity can be induced by combining rGM-CSF with rIFN-γ (30). Therefore, biological agents may differ in their ability to activate the tumoricidal properties of monocytes.

The i.v. administration of L-MTP-PE activated tumoricidal properties in the monocytes of 24 of the 28 patients studied.
This increased antitumor activity could not be explained by simple fluctuations in cytotoxic function because monocyte-mediated antitumor cytotoxicity in both normal donors and cancer patients did not change when sequentially studied over time (28). The optimal dose of L-MTP-PE for rendering monocytes tumor cytotoxic was 0.5-2.0 mg/m². Because the protocol schema called for each patient to have two dose escalations, the data were difficult to analyze. We therefore used three different analyses to determine the optimal dose (Figs. 1 and 2; “Results”). Future trials with a constant dose of L-MTP-PE may be able to define a narrower optimal biological dose, as we did for rIFN-γ (29). As observed with rIFN-γ, the optimal biological dose of L-MTP-PE was less than the maximum tolerated dose [L-MTP-PE MTD = 6 mg/m² (dose)].

Monocytes isolated from patients receiving doses of L-MTP-PE that exceeded the optimal dose could still be activated in vitro to lyse tumor cells and secrete IL-1 (data not shown). This is in contrast to monocytes obtained from patients receiving rIFN-γ at doses above the optimal biological dose where no response to in vitro stimuli could be generated (29). We therefore concluded that L-MTP-PE was not toxic to the monocytes at doses as high as 12 mg/m², the highest dose administered in this study.

Before the first i.v. infusion of L-MTP-PE, the monocytes of 8 of the 28 patients mediated high spontaneous antitumor activity (≥35%). The reason for this is unclear. Prior chemotheraphy with a drug capable of activating monocytes (e.g., Adriamycin) (31) is not responsible for the finding. Radiation can enhance the cytotoxic activity of human monocytes (32), and 7 of the 8 patients had previously had radiation therapy. One patient had radiation therapy only 18 days before the L-MTP-PE therapy. In addition, to radiation therapy, 2 of the 7 patients received rIFN-γ therapy 1 month before beginning the phase I trial with L-MTP-PE. The rIFN-γ exposure could have contributed to the high background (29). In these patients monocyte-mediated cytotoxic activity decreased following infusion with high doses of L-MTP-PE (Figs. 1 and 2). We have no explanation for this finding. This decrease could be due to the extravasation of activated monocytes from the peripheral blood into tissue parenchyma or, alternatively, to the stimulation of prostaglandin secretion, which can inhibit both monocyte-mediated cytotoxicity and IL-1 secretion (33). In three of the eight patients we monitored the secretion of IL-1 by monocytes in parallel with cytotoxic function. Monocytes of all three patients secreted IL-1 before the L-MTP-PE infusion but following therapy the secretion of IL-1 decreased in accordance with the cytotoxic values (Fig. 3; “Results”). These data further support the hypothesis that monocyte tumoricidal activity correlates with IL-1 secretion (34-36). We were unable to assay for TNF activity because of the small sample volume, but future studies will attempt to correlate TNF and IL-1 secretion patterns with the activation of tumoricidal properties in monocytes following L-MTP-PE administration.

In summary, the systemic administration of L-MTP-PE can render blood monocytes of cancer patients tumor cytotoxic. The optimal biological dose appears to be 0.5-2.0 mg/m²/dose. Since L-MTP-PE has no direct anti-proliferative effects on tumor cells, the objective of this therapy is to activate the host’s monocytes to kill the tumor cells. We therefore recommend that future phase I and phase II trials be conducted with a dose of 0.5-2.0 mg/m² rather than the maximum tolerated dose of 6 mg/m².

REFERENCES


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