Adjuvant Therapy for Osteosarcoma in Dogs: Results of Randomized Clinical Trials Using Combined Liposome-encapsulated Muramyl Tripeptide and Cisplatin[1]

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ABSTRACT

Two randomized, double-blind clinical trials in dogs with spontaneous appendicular osteosarcoma treated with combination chemoimmunotherapy are reported. In both trials, dogs without overt metastasis underwent complete amputation of the affected limb. In trial 1, 40 dogs were treated with cisplatin chemotherapy [(CDDP), 70 mg/m^2 i.v. every 28 days × 4]. Following CDDP, dogs without evidence of overt metastasis (n = 25) were randomized to receive liposome-encapsulated muramyl tripeptide phosphatidylethanolamine [(L-MTP-PE), 2 mg/m^2 i.v.) or placebo liposomes (lipid equivalent) twice weekly for 8 weeks. Of 14 dogs in the placebo group, 13 (93%) died of metastasis; the median survival time was 9.8 months. Of 11 dogs in the L-MTP-PE group, 8 (73%) developed metastasis; the median survival time was 14.4 months, which was significantly longer than that of the placebo group (P < 0.01). In trial 2, 64 dogs received CDDP (70 mg/m^2 i.v. every 21 days × 4) and were randomized to concurrently receive L-MTP-PE (2 mg/m^2 i.v.) twice or once weekly, or placebo liposomes once weekly for 8 weeks. Median survival times were 10.3, 10.5, and 7.6 months, respectively. There were no significant differences among the three treatment groups in trial 2. Survival times for dogs receiving L-MTP-PE in trial 1 were significantly longer than those for dogs in trial 2 that received four doses of CDDP concurrently with twice weekly L-MTP-PE (P < 0.04). The results of the first trial confirm our previous observation that L-MTP-PE has antimetastatic activity in dogs with osteosarcoma when given following amputation. The results of the second trial demonstrate that there is no survival advantage of administering L-MTP-PE concurrently with CDDP.

INTRODUCTION

Despite major advances in combination chemotherapy in human patients with osteosarcoma of the extremity, 35–40% of treated patients die of metastatic disease (1–4). Conventional cancer therapy may fail when heterogeneous clones of tumor cells emerge from the primary tumor to form metastases that are resistant to standard treatment. Furthermore, metastatic cells are usually more resistant to cytotoxic effects of chemotherapy (5). Macrophages and monocytes, when functionally activated, can destroy chemotherapy-resistant cancer cells *in vitro* (6–8). L-MTP-PE[3] has been shown to activate both human and canine monocytes, augmenting the tumoricidal effects of these cells *in vitro* and *in vivo* (9–16). It has been shown that L-MTP-PE or liposome-encapsulated muramyl dipeptide can activate macrophages *in situ*, which mediates regression of spontaneous metastases in a number of murine models: melanoma (17–19), fibrosarcoma (20), and liver carcinoma (21). In spontaneous canine osteosarcoma, L-MTP-PE following surgery significantly prolongs time to development of metastasis and survival compared to surgery alone (22). The results of phase I and II human clinical trials indicate that L-MTP-PE is well tolerated at doses known to induce desirable biological effects (11, 13, 23, 24).

The purpose of this study was to evaluate the effectiveness of L-MTP-PE in combination with chemotherapy for preventing or delaying the onset of distant metastasis in dogs with osteosarcoma. Canine osteosarcoma is a spontaneous malignancy which has micrometastasis at the time of diagnosis and is considered the best model for human osteosarcoma (25, 26). These tumors are histologically indistinguishable from human osteosarcoma; the vast majority are high-grade tumors and present with stage 2B disease. It is likely that all affected dogs have micrometastases at the time of diagnosis (25–28). The most commonly reported metastatic site in the dog is the lung. Despite various therapeutic regimens, most involving amputation, survival times remain short. Median survival times range

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[3] The abbreviations used are: L-MTP-PE, liposome-encapsulated muramyl tripeptide phosphatidylethanolamine; CDDP, cisplatin; MTP-PE, muramyl tripeptide phosphatidylethanolamine; TNF-α, tumor necrosis factor α.

from 3 to 6 months, and only about 10% of the dogs survive for 1 year or longer when treated with amputation alone (27, 28). Very few prognostic factors have been reported. In one study of 162 dogs, those younger than 5 years of age had a poorer prognosis than older dogs (28). Large tumor size was associated with the presence of tumor metastases at autopsy in untreated dogs (29). Primary tumors arising in the humerus (30) have been weakly associated with a poorer prognosis. No other factors are known to affect prognosis.

Chemotherapy protocols which utilize CDDP as the major drug have shown that median survival times will range from 9 to 11 months (30–34). The studies presented here were designed to treat micrometastasis using L-MTP-PE following (trial 1) or in combination (trial 2) with a course of CDDP chemotherapy. In addition, in trial 2 we compared once weekly to twice weekly administration of L-MTP-PE.

MATERIALS AND METHODS

Animals

One hundred eleven dogs with previously untreated, histologically confirmed primary osteosarcoma of the extremity, without radiographic evidence of distant metastasis, were studied. The evaluation included a complete blood count, serum biochemistry profile, urinalysis, and radiographs of the primary tumor and thorax. Only dogs without evidence of overt metastasis, in overall good health, and good candidates for amputation were admitted into this study. Written consent for entry into these trials was obtained from each dog's owner prior to treatment.

Treatment

Primary treatment was amputation of the affected limb. Amputations were done in a routine manner: complete forequarter amputation or hip disarticulation. A complete blood count, serum urea nitrogen concentration, creatinine concentration, and urinalysis were done prior to each CDDP chemotherapy. Dogs with pretreatment neutropenia (<3,000 neutrophils/μl) or thrombocytopenia (<75,000 platelets/μl) had their treatment delayed and were rechecked 7 days later to determine whether the neutropenia or thrombocytopenia had resolved. Within 24 h after surgery, dogs were started on CDDP chemotherapy (70 mg/m^2 i.v.) following standardized saline diuresis (31). All dogs were scheduled to receive a total of four doses of CDDP. All treatments were administered in accordance with a protocol approved by the University of Wisconsin Research Animal Resource Committee.

Trial 1. All dogs in this trial were from the patient population of the Veterinary Medical Teaching Hospital at the University of Wisconsin School of Veterinary Medicine. Dogs were scheduled to receive CDDP (70 mg/m^2 i.v.) every 28 days for a total of four treatments. One month after the completion of the last CDDP treatment (*i.e.*, 4 months after surgery), dogs were again evaluated for metastasis and, if they were free of any clinical evidence of metastatic disease, were randomized to receive either L-MTP-PE or placebo liposomes (lipid equivalent). The randomization procedure was a blocked random assignment method which changed after each group of four dogs were entered (35). All treatment assignments were double blind. The liposome preparation was given twice weekly for 8 weeks using a slow i.v. infusion over a 5–8-min period. The dose of L-MTP-PE was 2 mg/m^2 (500 mg phospholipids). This dose was based on previous studies in the dog (22).

Trial 2. This was a multi-institutional, centrally randomized (University of Wisconsin) clinical trial. L-MTP-PE or placebo was prepared and sent to participating institutions for each animal entered. All treatment assignments were double blind. Dogs were scheduled to receive CDDP (70 mg/m^2 i.v.) every 21 days for a total of four treatments. At the time of the first CDDP treatment, dogs were randomized to receive L-MTP-PE (2 mg/m^2 i.v.) either twice or once weekly, or placebo liposomes (lipid equivalent) once weekly, to begin 24 h after the first CDDP treatment. Liposomes were administered as described in trial 1.

Liposome Preparation

Lyophilized liposomes with or without MTP-PE (CGP 19835A lipid) were provided by Ciba-Geigy Limited (Basel, Switzerland). Liposomes were prepared from freeze-dried preparations by the addition of buffered saline, without calcium or magnesium, to vials containing dioleoyl-phosphatidylserine and 1-palmitoyl-2-oleoyl-phosphatidylcholine at a 3:7 molar ratio, with or without MTP-PE. The ratio of active ingredient (MTP-PE) to phospholipid was 1:250. After 1 min, the vial contents were agitated on a vortex mixer or vigorously shaken by hand for 1 min, and then the contents were diluted with buffered saline to a concentration of 25 mg lipid/ml. The preparation was then administered i.v. through a 10-μm filter.

Progress Examinations

In both trials, dogs were reexamined with routine physical examination and thoracic radiographs at 2-month intervals following the amputation. Physical and historical abnormalities were investigated as clinically indicated at each recheck visit. Patient evaluations continued as long as necessary to determine the metastasis-free interval and survival time for each dog. The metastasis-free interval was defined as the time from surgery to evidence of clinically detectable metastasis. Survival time was defined as the time from surgery to death or euthanasia due to advanced disease. Euthanasia was performed when requested by the dog's owner and when the dog's quality of life was considered severely diminished due to the advanced stage of the disease.

Statistical Considerations

The metastasis-free intervals and survival times were compared between the L-MTP-PE and placebo liposome groups in both trials. Survival times were also compared between dogs receiving L-MTP-PE in trial 1 and dogs receiving L-MTP-PE twice weekly in trial 2, and between dogs receiving placebo in trial 1 and those receiving placebo in trial 2. Metastasis-free interval and survival curves were generated by the Kaplan-Meier method and compared using the Breslow and Mantel-Cox tests of significance between survival curves. In addition, the Cox proportional hazards model was used to evaluate the effect of site of the primary tumor, sex, and age (<5 years old *versus* ≥ 5 years old) on metastasis-free intervals and sur-

vival times. These statistical tests adjust for dogs still alive or lost to follow-up at the time of analysis. A P value of <0.05 was considered significant.

RESULTS

The L-MTP-PE treatments were well tolerated by the dogs. The only consistent side effect was an elevation in the body temperature (1–2°C), lasting 1–4 h following the liposome injection. The temperature elevation was more pronounced in the L-MTP-PE groups. Body temperature returned to normal within 6 h after injection. The fever response was most apparent on the first treatment and then subsided on subsequent treatments.

Trial 1. Forty dogs were initially entered into this clinical trial. During the chemotherapy administration period, 15 dogs were excluded from randomization because 13 dogs developed metastasis and 2 died of other causes (1 died from a gastric torsion and 1 died of renal failure most likely related to the CDDP chemotherapy). Twenty-five dogs were randomized after the completion of 16 weeks of chemotherapy. Dogs eligible for randomization ranged in age from 1 to 12 years, with a median age of 7 years. There were 12 males and 13 females. Eleven dogs were randomized into the L-MTP-PE group and 14 were randomized into the placebo group. Of the 14 dogs in the placebo group, 13 (93%) died of metastasis and 1 died of an unrelated cause (neurological disease). The median metastasis-free interval for the dogs in the placebo group was 7.6 months, and the median survival time was 9.8 months. Four (29%) dogs survived for more than 1 year. In the L-MTP-PE group, 8 (73%) of 11 dogs developed metastasis. Two dogs died of unrelated causes (both euthanized for severe arthritis), one is still alive and free of metastasis, one is alive with metastasis, and one with metastasis was lost to follow-up. The median metastasis-free interval for the L-MTP-PE group was 11.2 months, and the median survival time was 14.4 months. Seven (64%) dogs survived for more than 1 year. Those dogs receiving L-MTP-PE had a significantly longer metastasis-free interval ($P < 0.035$) and survival time ($P < 0.01$) compared to dogs given placebo liposomes (Figs. 1 and 2). No effect on survival was observed with regard to site of the primary tumor, sex, or age.

Trial 2. Seventy-one dogs were entered into this study. Seven dogs were not available for evaluation for the following reasons: three dogs died of unknown causes within 3 weeks of entering the study, one of which had a necropsy which showed no metastatic disease nor was the cause of death determined, and the owners of the other two dogs declined a necropsy; one died of gastric torsion within 3 weeks of entering the study; two dogs were removed from the study by their owners within 2 weeks of being entered; and one dog was removed from the study due to reclassification of its tumor as a chondrosarcoma. The remaining 64 dogs ranged in age from 1.5 to 14 years, with a median age of 8 years. There were 25 males and 39 females. Twenty-one dogs were randomized to receive L-MTP-PE twice weekly, 21 received L-MTP-PE once weekly, and 22 received placebo liposomes once weekly. There were no significant differences among the three groups with regard to metastasis-free intervals (Fig. 3) or overall survival times (Fig. 4). A total of 58 dogs completed all four doses of chemotherapy; 6 dogs did not receive all four doses because they developed metastasis before completion of the planned chemotherapy. Median metastasis-free intervals for the three groups were 7.5, 6.3, and 5.8 months, respectively (Fig. 3). Median survival times for the three groups were 10.3, 10.5, and 7.6 months, respectively (Fig. 4).

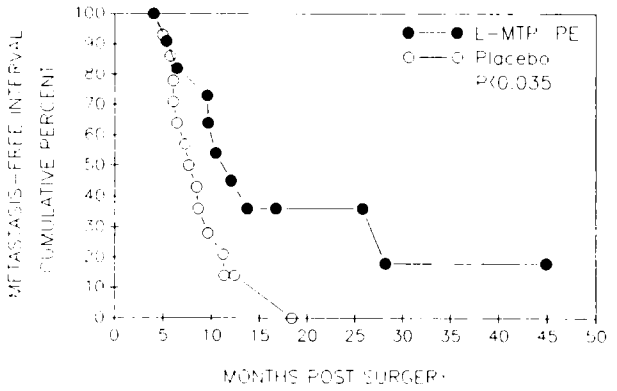

Fig. 1 Metastasis-free intervals for dogs with osteosarcoma treated with surgery and CDDP, then randomized to L-MTP-PE or placebo liposomes. Dogs receiving L-MTP-PE had significantly longer metastasis-free intervals ($P < 0.035$).

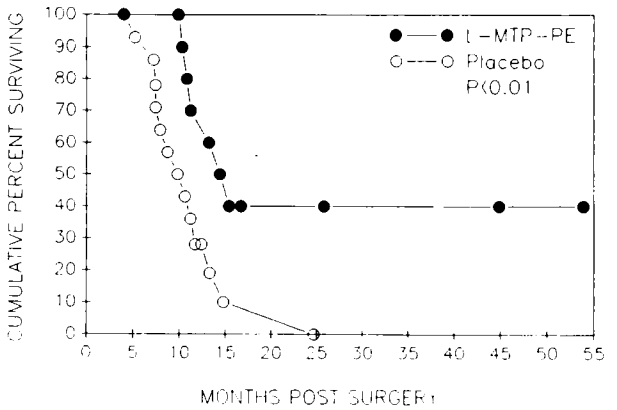

Fig. 2 Survival times for dogs with osteosarcoma treated with surgery and CDDP, then randomized to L-MTP-PE or placebo liposomes. Dogs receiving L-MTP-PE had significantly longer survival times ($P < 0.01$).

In the twice weekly L-MTP-PE group, 19 (90%) of 21 dogs developed metastasis, 1 died of an unrelated cause (idiopathic hypertrophic cardiomyopathy) with no evidence of metastasis, and 1 is alive and free of disease; 7 (33%) dogs survived for 1 year or longer. In the once weekly L-MTP-PE group, 18 (86%) of 21 dogs developed metastasis, 3 dogs with metastasis died of unrelated causes (renal failure, gastric ulcer, and gastric torsion), 1 dog with metastasis was lost to follow-up, and 3 are alive and free of disease; 7 (33%) dogs survived for 1 year or longer. In the placebo group, 17 (77%) of 22 dogs developed metastasis, 2 dogs died of renal disease with no evidence of metastasis, and 3 are alive and free of disease; 9 (41%) dogs survived for more than 1 year.

There was no difference with regard to survival among the three treatment groups in trial 2, thus the groups were combined

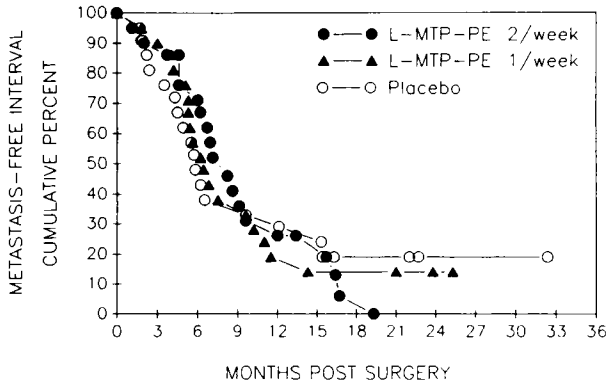

Fig. 3 Metastasis-free intervals for dogs with osteosarcoma treated with surgery followed by CDDP and L-MTP-PE or placebo liposomes. There were no significant differences among the three treatment groups.

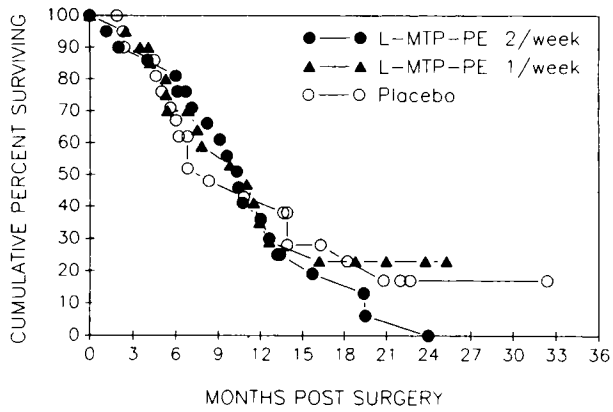

Fig. 4 Survival times for dogs with osteosarcoma treated with surgery followed by CDDP and L-MTP-PE or placebo liposomes. There were no significant differences among the three treatment groups.

for the purpose of evaluating the effect of site of the primary tumor, body weight, sex, and age on metastasis-free interval and survival. The median metastasis-free interval for all 64 dogs was 6.6 months, and the median survival time was 10.3 months. No effect from primary tumor site was observed, with the exception of osteosarcoma of the scapula which had a significantly shorter metastasis-free interval and survival time (3 and 3.5 months, respectively); however, there were only two dogs with tumors arising at this location. The age, sex, or neuter status of the dog had no relationship to survival.

Trial 1 *versus* Trial 2. Survival times of the 11 dogs receiving twice weekly L-MTP-PE in trial 1 were compared to those of the 18 dogs in trial 2 receiving twice weekly L-MTP-PE concurrently with four doses of CDDP. The dogs in trial 1 were not randomized until 4 months after surgery, thus this trial only included dogs that were free of metastasis at 4 months. There were three dogs in trial 2 that received L-MTP-PE twice weekly, but were not free of metatasis at 4 months. To eliminate bias in comparing survival times for dogs in trial 1 to dogs in trial 2 that received L-MTP-PE twice weekly, we excluded those three dogs only from this analysis. Median survival times were 14.4

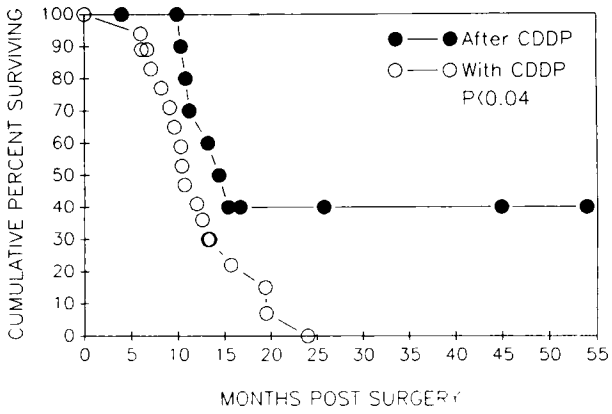

Fig. 5 Survival times for dogs with osteosarcoma receiving L-MTP-PE twice weekly for 8 weeks either following or concurrently with four doses of CDDP. Dogs receiving L-MTP-PE after CDDP therapy had significantly longer survival times ($P < 0.04$).

and 10.6 months for the dogs in trials 1 and 2 that were free of metastasis at 4 months after surgery, respectively ($P < 0.04$; Fig. 5). Survival times of the 14 dogs receiving twice weekly placebo liposomes in trial 1 were compared to those of 16 dogs in trial 2 that received once weekly placebo liposomes concurrently with four doses of CDDP. Using the same rationale for exclusion for this comparison (*i.e.*, comparing only dogs that were free of metastasis at 4 months after surgery), five dogs in trial 2 were excluded from this comparison because they had developed metastasis prior to 4 months after surgery, and one dog was excluded because it died of renal failure at 1.9 months after surgery. There was no difference between the two placebo groups with respect to median survival times, which were 9.8 (trial 1) and 10.8 (trial 2) months.

DISCUSSION

The usefulness of canine osteosarcoma as a comparative model for human osteosarcoma has recently been reported (26). Approximately 8000 dogs with osteosarcoma are diagnosed annually in the United States *versus* 2100 human patients with osteosarcoma. The biological behavior of osteosarcoma in dogs is similar to that in humans, and the evaluation of adjuvant chemotherapy or immunotherapy in dogs may have direct application to the management of human osteosarcoma.

The results in trial 1 confirm earlier observations that L-MTP-PE has antitumor activity in dogs with spontaneously occurring osteosarcoma. The results of this study compare favorably to our previous study evaluating amputation alone *versus* amputation combined with L-MTP-PE treatment (22). In that trial, dogs receiving L-MTP-PE had a median survival time of 7.3 months following amputation compared to 2.5 months for dogs treated by amputation alone (22). In trial 1 of the current report, the addition of CDDP chemotherapy, without L-MTP-PE, following surgery increased the median survival time to 9.8 months, similar to the end results described in other canine osteosarcoma studies using CDDP or carboplatin adjuvant to amputation (30–34). In the present study, the addition of L-MTP-PE following CDDP chemotherapy increased the me-

dian survival time to 14.4 months. Although the number of dogs in this treatment group is small ($n = 11$), we note that 14.4 months is the longest reported median survival time for dogs with osteosarcoma treated by amputation and any form of adjuvant therapy.

In trial 1, 13 (32%) of 40 dogs initially entered into the study developed metastasis prior to the time of randomization (*i.e.*, prior to 4 months after surgery). The purpose of trial 2 was to administer L-MTP-PE shortly after surgery with the expectation that the concurrent administration of CDDP and L-MTP-PE would have a greater antitumor activity than when given sequentially, thereby preventing the development of metastasis early in the postoperative period as seen in trial 1. In trial 2, however, the antitumor activity of L-MTP-PE noted in trial 1 was not observed. We conclude that there is no survival advantage when L-MTP-PE is administered concurrently with CDDP. Because of the lack of effect of L-MTP-PE in trial 2, we were unable to compare twice weekly to once weekly administration.

To further clarify the benefits of L-MTP-PE administration following CDDP (trial 1) administration compared to concurrent administration of L-MTP-PE with CDDP (trial 2), we compared survival times for the dogs in trial 1 that received L-MTP-PE (2 mg/m^2 i.v., twice weekly for 8 weeks) to the survival times for dogs in trial 2 that received L-MTP-PE (2 mg/m^2 i.v., twice weekly for 8 weeks) concurrently with four doses of CDDP. For this comparison, we excluded dogs in trial 2 that had developed metastasis prior to the time that dogs in trial 1 were randomized (*i.e.*, prior to 4 months after surgery), so that all dogs being compared were free of metastasis at 4 months following surgery. In support of our hypothesis that there is no survival advantage of concurrent administration of L-MTP-PE and CDDP, we found that dogs receiving L-MTP-PE after CDDP administration had significantly longer survival times than those dogs receiving L-MTP-PE concurrently with CDDP (Fig. 5). To address the potential concern that there might be a difference in survival times for dogs receiving CDDP every 28 days (trial 1) *versus* dogs receiving CDDP every 21 days (trial 2), we compared survival times of the dogs receiving placebo liposomes in these two trials. Again, dogs in trial 2 that developed metastasis prior to 4 months after surgery were excluded from this analysis. No difference in survival was noted between the two placebo groups, suggesting that the interval of CDDP administration did not affect survival times in this study.

L-MTP-PE also has antitumor activity in humans with osteosarcoma. In a Phase II study reported by Kleinerman and colleagues (36–38), 16 human osteosarcoma patients received L-MTP-PE at 2 mg/m^2 i.v. twice weekly for 12 weeks and then once weekly for an additional 12 weeks. For those 16 patients receiving 24 weeks of L-MTP-PE, the median disease-free interval significantly exceeded that of a historical control group of 21 patients receiving chemotherapy alone (9 and 4.5 months, respectively; $P < 0.03$). In the control group (chemotherapy alone), only 15% of the patients were disease-free at 10 months compared to 49% in the 24-week L-MTP-PE group. Further evidence of antitumor activity induced by L-MTP-PE was reported in five human osteosarcoma patients with metastasis to the lungs (24). Histological evaluation of surgically removed lung metastases from L-MTP-PE-treated patients revealed peripheral fibrosis surrounding the pulmonary lesion in four of five patients. In all five patients, infiltration of the tumor with mononuclear cells was noted. Immunohistochemistry studies confirmed that these cells were activated macrophages. An additional observation was that the tumor malignancy grade changed from a "high grade" prior to L-MTP-PE to a "low-grade" after treatment in two of five patients treated (24).

The influence of chemotherapy administration on *in vitro* monocyte activation by L-MTP-PE was studied in children with osteosarcoma (39). Single agent chemotherapy with CDDP, high-dose methotrexate, cyclophosphamide, or doxorubicin did not interfere with the *in vitro* activation of monocytes from these patients. There was a suggestion of enhanced monocyte activation following doxorubicin. However, when both doxorubicin and cyclophosphamide were administered together on the same day, profound suppression in monocyte activation was observed (39). *In vitro* studies have shown that increasing concentrations of doxorubicin did not alter human monocyte activation or release of TNF-α and interleukin 1 (39). In the dog, it has been demonstrated that doxorubicin alone will increase monocyte cytotoxicity at 3 and 7 days after administration (15). Furthermore, when dogs were administered L-MTP-PE in combination with doxorubicin, monocyte cytotoxicity was greatly enhanced as well as serum TNF-α activity.

L-MTP-PE administered in combination with ifosfamide in human osteosarcoma patients was able to induce monocyte tumoricidal activity as well as cytokine release (TNF-α, interleukin 6, C-reactive protein, and neopterin). The administration of ifosfamide with L-MTP-PE did not suppress any of the responses mentioned above. Ifosfamide toxicity was not increased nor were delays in ifosfamide treatment necessary when combined with L-MTP-PE (40). In a recently completed study of dogs with splenic hemangiosarcoma, L-MTP-PE was given concurrently with chemotherapy (doxorubicin and cyclophosphamide). Dogs receiving L-MTP-PE and chemotherapy had significantly longer survival times than those receiving placebo liposomes and chemotherapy, 9.1 and 4.7 months, respectively ($P = 0.029$; Ref. 41).

It appears that both timing and type of chemotherapy can affect the antitumor activity of immunotherapy agents such as L-MTP-PE. In support of our findings in trial 2, it has been reported that in the B16-F10 mouse melanoma model, CDDP, when given concurrently with L-MTP-PE, partially negated the beneficial effect of L-MTP-PE on the control of pulmonary metastases (42). We conclude that L-MTP-PE has significant antitumor activity when administered alone; however, when given concurrently with CDDP the survival advantage of L-MTP-PE is not observed. Additional studies are needed to develop more effective chemotherapy regimens that can be administered with L-MTP-PE to obtain even more desirable control of metastasis in patients with disseminated cancer. The results of our trials in a spontaneous large animal model provide important information toward attaining that goal.

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