**In Vivo Modulation of Macrophage Tumoricidal Activity by Oral Administration of the Liposome-encapsulated Macrophage Activator CGP 19835A**

**Simon Tanguay, Corazon D. Bucana, Michael R. Wilson, Isaiah J. Fidler, Andrew C. von Eschenbach, and Jerald J. Killion**

**Departments of Cell Biology [S. T., C. D. B., M. R. W., I. J. F., J. J. K.] and Urology [A. C. v. E.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030**

**ABSTRACT**

The present study evaluated the *in vivo* biological activity of synthetic muramyl tripeptide, CGP 19835A, when encapsulated into phosphatidylcholine liposomes (POPC-19835A) and administered as a p.o. immunomodulator to BALB/c mice. Liposomes were rapidly absorbed in the intestine and reached the systemic circulation within 4 h. Alveolar macrophages harvested from the lungs of mice 24 h after a single p.o. feeding of POPC-19835A were tumoricidal toward syngeneic murine renal cell carcinoma target cells. Repeated daily feedings with POPC-19835A generated sustained activation of the alveolar macrophages. Activation of peritoneal macrophages to the tumoricidal state required at least three daily feedings of POPC-19835A. In *vitro* studies demonstrated the release of tumor necrosis factor-α and interleukin-6 by macrophages activated by POPC-19835A in the presence of γ-interferon. Interleukin-1 and nitric oxide were not induced in macrophages by this liposomal preparation. Daily administration of POPC-19835A after i.v. injection of renal cell carcinoma tumor in BALB/c mice inhibited the development of experimental lung metastasis and confirmed the potential role of long-term immunomodulation with this new p.o. immunomodulator.

**INTRODUCTION**

Systemic activation of macrophages to the tumoricidal state can be achieved by administration of synthetic liposome-encapsulated activators, such as the analogues of muramyl dipeptide (1–5). We have demonstrated that i.v. injection of liposome-encapsulated MTP-PE causes regression of established metastases of melanoma and RENCA in syngeneic mice, as well as osteosarcoma in dogs (6–9). Also, recent reports of a phase II trial in patients with osteosarcoma demonstrated that i.v. administration of liposome-encapsulated MTP-PE resulted in systemic activation of tissue macrophages after p.o. administration. However, encapsulation of MTP-PE into multilamellar liposomes would enhance the distribution and delivery of this macrophage activator to tissue macrophages after p.o. administration. It is not known whether encapsulation of MTP-PE into multilamellar liposomes would enhance the distribution and delivery of this activator to tissue macrophages after p.o. administration. However, liposomes composed of POPC are phagocytosed by macrophages and are stable in the presence of serum (14). We therefore evaluated the *in vivo* modulation of macrophage function by POPC-19835A when given to mice by repeated p.o. feedings. We demonstrate that p.o. feedings of POPC-19835A resulted in systemic activation of tissue macrophages associated with the release of cytokines and in inhibition of experimental lung metastasis of the RENCA tumor in syngeneic mice.

**MATERIALS AND METHODS**

**Animals.** Specific-pathogen-free BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). The mice were matched for age (6–10 weeks) and sex and used according to institutional guidelines. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the United States Department of Agriculture, Department of Health and Human Services, and the standards and regulations of the NIH.

**Tumor Cell Culture.** The spontaneous renal adenocarcinoma, RENCA (15), was grown as a monolayer culture in CMEM. The complete medium was free of endotoxin, as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2} in air. All cell cultures were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K virus, encephalitis virus, lymphocyte choriomeningitis virus, astrovirus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

**Reagents.** CGP 19835A is a lipophilic muramyl dipeptide analogue. This macrophage activator and its encapsulating phospholipid, [L-a-1-palmitoyl-2-deoxy-POPC], were supplied as dry lyophilisate (2 mg active material, CGP 19835A; 250 mg lipidi) by Ciba-Geigy, Ltd., Basel, Switzerland. MLVs were prepared by hydrating the lyophilized material in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free HBSS for 1 min at room temperature, then by vigorous shaking for 6 min using a vortex shaker at a high setting. Recombinant mouse IFN-γ (10\textsuperscript{4} units/mg) were obtained from Genzyme (Cambridge, MA). TNF-α (2 × 10\textsuperscript{6} units/mg) were purchased from Gibco BRL (Grand Island, NY). Thioglycolate broth was obtained from Baltimore Biological Laboratories (Cockeysville, MD). Phenol-extracted Salmonella LPS was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents (with the exception of LPS) contained less than 0.125 ng/ml endotoxin.

**p.o. Administration of POPC-19835A.** Mice were fed (without anesthesia) using a curved rigid feeding tube. The tube was inserted into the esophagus and a 0.2-ml solution of HBSS containing the liposome preparation was deposited into the stomach. This procedure was well tolerated by the animals.

**Organ Distribution of [\textsuperscript{3}H]POPC-19835A.** To determine organ distribution of the liposomes, 2 μCi of [\textsuperscript{3}H] phosphatidylcholine (42 nmol; DuPont, Albany, NY) were incorporated into 250 μCi POPC-19835A. Mice were fed 2.5 μCi [\textsuperscript{3}H]POPC-19835A (containing about 100,000 cpm) in 0.2 ml of HBSS. Groups of three mice each were killed at time intervals from 15 min to 24 h after feeding. At each time point, the lungs, stomach, intestine, liver, spleen, kidneys, and 1 ml of blood were harvested. The organs were washed in PBS and shaken for 16 h at 4°C in chloroform:methanol:1 n HCl (1:2:0.8, respec-

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3 To whom requests for reprints should be addressed, at the Department of Cell Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

4 The abbreviations used are: RENCA, renal cell carcinoma; CGP 19835A, N-acetyl-muramyl-l-tosylalanyl-2-(2′-dipalmitoyl)-sn-glycerol-3′-phosphorylethylamide; AM, alveolar macrophage(s); HESS, Hanks' balanced salt solution; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MLV, multilamellar vesicle; MTP-PE, muramyl tripeptide phosphatidyethanolamine; NBD-PE, N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidyethanolamine; PBS, phosphate-buffered saline; PEM, peritoneal exudate macrophage; PM, peritoneal macrophage(s); POPC, phosphatidylcholine; TNF, tumor necrosis factor; CMEM, Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids; MLV-HBSS, medium plus liposomes containing HBSS; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; dThd, thymidine.

Earlier work demonstrated the ability of free-form MTP-PE to activate alveolar macrophages in mice after repeated p.o. feedings (13). It is not known whether encapsulation of MTP-PE into multilamellar liposomes would enhance the distribution and delivery of this activator to tissue macrophages after p.o. administration. However, liposomes composed of POPC are phagocytosed by macrophages and are stable in the presence of serum (14). We therefore evaluated the *in vivo* modulation of macrophage function by POPC-19835A when given to mice by repeated p.o. feedings. We demonstrate that p.o. feedings of POPC-19835A resulted in systemic activation of tissue macrophages associated with the release of cytokines and in inhibition of experimental lung metastasis of the RENCA tumor in syngeneic mice.
ative ratio) to extract the lipid from the different organs. The tubes were then centrifuged at 2500 rpm for 10 min. Methanol was added until only one phase was present after centrifugation. One ml of the supernatant was added to the same amount of chloroform and 1 N HCl. The samples were shaken for 1 min and centrifuged. The organic phase was isolated and dried in scintillation vials with a stream of nitrogen gas. Radioactivity was measured in a Beckman model LS 3800 liquid scintillation counter (Beckman Fullerton, CA). The total amount of radioactivity recovered from each organ was then calculated. Thin layer chromatography was also performed using POPC as a standard to detect breakdown products of the radiolabeled phosphatidylcholine liposomes extracted from tissues. This technique revealed that about 35% of the radioactive label was intact as phosphatidylcholine after 4 h (data not shown).

Organ Distribution of Fluorescent Liposomes. Fluorescent liposomes were prepared by incorporating 0.5% of the nonexchangeable fluorescent marker N-4-nitrobenzo-2-oxa-1,3-diazolephosphatidylethanolamine into POPC-19835A liposomes (16). Mice were fed 2.5 μM concentrations of the fluorescent liposomes. Groups of two mice were bled and killed 1, 4, and 24 h after the p.o. feeding. At each time point, samples of the lungs, stomach, intestine, and liver were harvested. The blood samples from the mice killed at each time point were pooled. The tissue samples were placed in OCT compound (Miles Laboratories, Naperville, IL) and frozen in liquid nitrogen; then 8-μm cryostat sections were fixed in 4% paraformaldehyde in PBS for 2 min. The sections were mounted with glyceral/PBS containing propyl gallate, and fluorescence was examined in a Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a fluorescence exciter/barrier filter set. Images were recorded by digitizing them by using a cooled CCD Optronics Tcc 470 camera (Optronics Engineering, Goleta, CA) linked to a computer and a Sony digital color printer (Sony Corporation, Tokyo, Japan).

Collection and Cultivation of Mouse Macrophages. AMs were harvested by a tracheobronchial lavage. Mice were anesthetized by an i.p. injection of 0.25 ml sodium pentobarbital (10 mg/ml), washed with 70% alcohol, placed in a laminar flow hood, and exsanguinated by severing the left renal artery. This procedure minimized pulmonary edema and trapped blood in the lungs. The chest cavity was opened, and a blunt 20-gauge needle was inserted into the trachea and fixed with a silk suture. The lungs were lavaged with 0.8 ml of HBSS prewarmed to 37°C. This process was repeated 10 times to yield about 1.5—2 × 10^6 AMmouse. Resident PMs were harvested from the mice by peritoneal lavage with 10 ml of HBSS. PEMs were collected by peritoneal lavage from mice given i.p. injections of 2 ml of thioglycolate broth 4 days before harvest (16). The cell suspensions were washed with HBSS, and 1 × 10^6 cells in serum-free medium were plated into 38-mm^2 wells of a microtiter plate. Nonadherent cells were removed 2 h later. At that time, >95% of the adherent cells were mononuclear phagocytes according to morphological and phagocytic criteria (17).

In Vivo and In Vitro Activation of Mouse Macrophages. For in vivo activation of either AM or PM, normal BALB/c mice were fed different amounts of POPC-19835A. The macrophages were harvested 20—24 h after the last p.o. feeding. Macrophages (1 × 10^6) in serum-free media were plated into 38-mm^2 wells of a microtiter plate. Nonadherent cells were removed after 2 h of incubation at 37°C and radiolabeled target cells were added. PEM from normal BALB/c mice were incubated at 37°C in vitro for 20—22 h with 0.2 ml of control medium or MLV-HBSS or CPG 19835A. LPS (1 mg/ml) was added as a positive control. Liposomes were suspended in medium with or without IFN-γ (10 units/ml). After the incubation period, macrophage cultures were thoroughly washed, and 1 × 10^6 [3H]TdR-labeled target cells were added as described below.

In Vitro Assay of Macrophage-mediated Cytotoxicity. Macrophage-mediated cytotoxicity was assessed by measuring the release of radioactivity from target cells as described previously (14, 16). RENCA cells in exponential growth phase were incubated for 24 h in CMEM containing 0.6 μCi/ml [3H]TdR (2 Ci/mmol; ICN Biomedicals, Costa Mesa, CA). The cells were washed four times with HBSS to remove unbound radioisotope and then harvested by a brief trypsinization. The cells were washed and resuspended in CMEM, and 1 × 10^6 cells were added to the macrophages (effector:target ratio, 10:1). After 72 h of cocultivation, the cultures were washed twice with HBSS and the remaining adherent and viable target cells were lysed with 0.1 ml of 0.1 N KOH. The lysates were harvested with a Harvester 96 (Tomtec, Orange, CT) and counted in a liquid scintillation counter. Cytotoxicity was calculated as:

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

where A was the cpm of target cells cultured with control macrophages and B was the cpm of target cells cultured with test macrophages.

Experimental Lung Metastasis Model. Cultured RENCA cells (70% confluent) were given fresh medium 24 h before harvest. The cells were rinsed in Ca^2+ and Mg^2+-free HBSS and treated for 2 min with a 0.25% trypsin-0.02% EDTA solution. The flask was tapped, and the cells were pipetted to produce a single cell suspension and then filtered through a 53 μm Nitex filter (TETKO, Lancaster, NY) to remove any large clumps of cells. The cells were then washed in HBSS and their viability was ascertained by trypan blue exclusion. Only suspensions with >95% viability were used for in vivo studies. The cell suspension was kept at 4°C.

RENSA cells were injected i.v. into the lateral tail vein of BALB/c mice. The tumor inoculum used (7.5 × 10^5-2.5 × 10^6 cells in 0.2 ml) resulted in lung metastases in 100% of the animals.

Liposome Therapy: Experimental Design. Therapy was started 3 days following the i.v. injection of RENCA tumor cells. Treatment consisted of p.o. feeding with either HBSS alone, empty liposomes (MLV-HBSS), or POPC-19835A-naphtyl ether (250 μg CPG 19835A). Groups of five mice were fed three times a week (3 on 3 consecutive days), except for one group, which received POPC-19835A daily. Treatment was administered for 3 weeks, and mice were killed on day 30 or when the control mice became moribund.

Assay for TNF-α. The presence of TNF-α in the supernatant of activated macrophages was assayed by lysis of the TNF-sensitive mouse fibroblast L-929 cell line. L-929 cells (5 × 10^3) were plated into 96-well microtiter plates. Fourteen h later, the nonadherent cells were removed and culture supernatants of activated macrophages were added to the target cell monolayers. As control groups, target cells were incubated in medium alone (no TNF-α) or medium containing various concentrations of recombinant TNF-α. Samples were incubated for 72 h, and the viability of the L-929 cells was assayed by a MTT-based colorimetric assay. MTT at a final concentration of 500 μg/ml was added to each well for 90 min at 37°C. Culture supernatants were then removed, and 100 ml of dimethyl sulfoxide was added. Absorbance at 570 nm was read on a microtiter plate reader (Bio-Rad Corp., San Francisco, CA). The percentage of lysis was calculated as described above, where A represents the absorbance of target cells cultured with medium alone and B represents the absorbance of target cells cultured with test supernatants or recombinant TNF-α.

Assay for IL-1. The D10.G4.1 bioassay for IL-1 has been described in detail (19, 20). Briefly, 1 × 10^6 D10 cells were cultured in the presence of 2.5 μg/ml concanavalin A and various dilutions of test supernatants for 48 h. [3H]TdTd (0.2 μCi/ml) was added to each well 18 h prior to harvest of the cells, and incorporated thymidine was measured by a scintillation counter.

Assay for IL-6. IL-6 secretion was assayed by enzyme-linked immunosorbent assay using the technique described by Meager (21). Pairs of IL-6 antibodies were obtained from Pharmingen (San Diego, CA). The amount of IL-6 of each sample was determined by comparison to a standard curve.

Nitric Oxide Production. Since nitrite is the stable oxidation product of NO, the nitrite concentration in culture supernatants was determined by the microplate assay described by Ding et al. (22). Briefly, supernatant samples were added to an equivalent volume of Griess reagent (1% sulfanilamide and 1% naphthylenediamine dihydrochloride in 2.5% H_3PO_4) at room temperature for 10 min. Absorbance at 540 nm was monitored with a microplate reader. The concentration of nitrite was determined by comparison with a standard solution of sodium nitrite in medium.

Statistical Analysis of Data. Differences in the median number of lung metastases between groups were compared using the Mann-Whitney test. Significant differences in in vitro cytotoxicity mediated by AM and PM were calculated using Student’s t test.

RESULTS

Distribution of POPC-19835A Liposomes. Groups of mice were fed with radiolabeled liposomes and organs were harvested 0.25, 1, 4,
and 24 h later. As shown in Table 1, the liposomes were rapidly absorbed in the intestine of mice with maximal uptake detected 1 h after the feeding. A slowly increasing amount of radioactivity in the liver, with a peak at 4 h, correlated with the kinetics of absorption in the intestine, suggesting rapid uptake of the liposomes with systemic distribution. Minimal radioactivity (above background level) could be detected in the kidneys and the lungs at 4 h. No detectable counts were found in the spleen or blood (data not shown).

To confirm the presence of liposomes in the systemic circulation, we fed mice liposomes that contained a stable, nonexchangeable fluorescent label (N-4-nitrobenzo-2-oxa-1,3-diazole-POPC 19835A; 2.5 μM). The organ distribution of the liposomes confirmed the

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<th>Table 1</th>
<th>In vivo distribution of POPC-19835A</th>
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<td><strong>Mice were fed 2.5 μM (20 μg) POPC-19835A labeled with [3H]phosphatidylcholine. The distribution of the liposomes into different organs was determined after 0.25, 1, 4, and 24 h.</strong></td>
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<tr>
<td></td>
<td>0.25 h</td>
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<tr>
<td>Stomach</td>
<td>1530 ± 645</td>
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<td>Intestine</td>
<td>15107 ± 712</td>
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<td>Liver</td>
<td>176 ± 154</td>
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<td>Lungs</td>
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Fig. 1. Mice were fed fluorescent (NBD) liposomes and samples of their intestine (A, B), liver (C, D), lungs (E, F), and blood (G, H) were harvested 4 h later and evaluated under phase microscopy (B, D, F, and H) or fluorescent microscopy (A, C, E, and G). A-F, × 480; G, H, × 680.
We then evaluated whether the tumoricidal activity of PM occurred as a consequence of repeated stimulations with POPC-19835A or whether PM required a period of 72 h before becoming fully activated. When mice were fed once with POPC-19835A, no significant tumoricidal activity was observed from PM harvested 72 h later. However, three consecutive daily feedings resulted in 33% cytotoxicity against RENCA target cells.

Eradication of Experimental Lung Metastasis. To evaluate the efficacy of activated AM in eradicating RENCA lung metastases, we injected BALB/c mice i.v. with different numbers of RENCA tumor cells. As shown in Table 3, for each cell inoculum used, neither HBSS nor MLV-HBSS administered three times per week affected the development of lung metastases. On the other hand, p.o. administration of POPC-19835A did inhibit the growth of lung metastases. Indeed, both three and seven treatments per week gave similar results in animals with a small tumor burden. However, as the tumor burden was increased (1.25 and 2.5 × 10⁴ cells), daily treatments were required to significantly reduce the number of tumor nodules.

In Vitro Activation of PEM. The cellular properties associated with activation of macrophages to the tumoricidal state by POPC-19835A were studied in vitro. PEM were incubated in medium alone

Findings described above. Fluorescent liposomes were present at all time points on the mucosa of the stomach wall (data not shown). Fig. 1 illustrates the presence of the N-4-nitrobenzo-2-oxa-1,3-diazole liposomes in the intestine, liver, lungs, and blood 4 h after p.o. administration. Fig. 1, G and H shows a blood monocyte with a strong fluorescent signal.

In Vivo Activation of Alveolar and Peritoneal Macrophages. We first evaluated whether p.o. administration of POPC-19835A could result in in situ activation of AM to a tumoricidal state. Groups of mice were fed with different amounts of POPC-19835A (0.5, 1.5, 2.5, and 5.0 μM). AMs were harvested 24 h later, and their ability to lyse RENCA tumor cells was measured in vitro. The results shown in Fig. 2 demonstrate that the activation of AM was dose dependent, with increasing cytotoxicity ranging from 19% (0.5 μM; 4 μg) to a maximal effect of 56% (2.5 μM; 20 μg). Administration of 2.5 μM MLV-HBSS p.o. did not activate AM to a tumoricidal state (data not shown).

We next determined the duration of tumoricidal activity of AM following a single dose of POPC-19835A. AMs were harvested from mice 1, 3, 5, and 7 days after the p.o. feeding of 2.5 μM POPC-19835A and tested for cytotoxic activity against RENCA target cells. Fig. 3 illustrates the decay of macrophage-mediated cytotoxicity as a function of time. Maximal cytotoxicity was present 24 h after administration of POPC-19835A, and it was decreased to baseline activity within 4–5 days.

We therefore studied the effect of repeated p.o. feedings of POPC-19835A using the feeding schedule for free-form MTP-PE (13). Mice were fed daily for 1, 2, or 3 days. Both alveolar and peritoneal macrophages were harvested. Table 2 shows that the cytotoxicity of AM was not enhanced by repeated p.o. administrations. Maximal activation was achieved after a single dose (51%), and cytotoxicity remained elevated during the time of repeated p.o. feedings. In addition, continued feeding of the mice for as long as 7 days resulted in a stable level of tumoricidal activity by AM (data not shown). PMs were not activated after a single p.o. feeding of 2.5 μM; whereas after 3 consecutive feedings, 32% cytotoxicity against RENCA target cells was obtained (P < 0.01, compared to normal PM).
The rationale for using liposomes as carriers for biological response modifiers is the increased delivery to the reticuloendothelial system (14, 26, 27). The majority of the POPC-19835A liposomes were absorbed within 4 h of p.o. administration. This access to the systemic circulation was confirmed by the use of both radiolabeled and fluorescent liposomes, which showed the presence of these liposomes in the blood with subsequent localization to the lungs. As a result, p.o. administration of POPC-19835A generated rapid in situ activation of AM. Maximal activation of AM was achieved with 20 μg of liposome-encapsulated CGP 19835A within 24 h of the p.o. feeding. The progressive decay in the tumoricidal activity of the AM over the next 5 days was prevented by repeated administration.

Administration of POPC-19835A p.o. also activated PM, whereas in a previous study i.v. administration of liposome-encapsulated CGP 19835A did not activate PM (13). This finding confirms the importance of route of administration in in vivo activation of macrophages. However, repeated feedings were required to induce the tumoricidal activity of these macrophages. Previously, p.o. administration of free-form MTP-PE required a latent period (48–72 h) before achieving PM activation (13). The p.o. administration of the liposome-encapsulated MTP-PE did not require this latent period, perhaps because of different distribution or different uptake of the liposomes by circulating monocytes. Collectively, these data suggest a potential therapeutic benefit of repeated p.o. administration.

The cellular mechanisms associated with regression of the RENCA tumor are unknown, but the antitumor effects of macrophage activation can be enhanced by cytokines (8, 28, 29). However, repeated feedings were required to induce the tumoricidal activity of these macrophages. Previously, p.o. administration of POPC-19835A generated rapid in situ activation of AM. Maximal activation of AM was achieved with 20 μg of liposome-encapsulated CGP 19835A within 24 h of the p.o. feeding. The progressive decay in the tumoricidal activity of the AM over the next 5 days was prevented by repeated administration.

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Therapy of experimental RENCA lung metastases with POPC-19835A proved to be, as expected, more effective when the tumor burden was small. Furthermore, continuous daily treatments gave the best results, suggesting a benefit from the presence of a constant level of macrophage activation. A recent phase II clinical trial in children with osteosarcoma demonstrated a significantly longer time to relapse of macrophage activators in long-term treatment of patients.

The route of administration of immunomodulators may influence the trafficking and responses of host immune cells. For example, it has been shown that p.o. administration of bacterial immunomodulators enhances the movement of intestine lymphocytes to the lungs in preference to other organs (33). Also, the i.v. administration of macrophage activators resulted only in tumoricidal activation of AM, whereas i.p. administration activated only PM (13). However, our results indicate that p.o. administration results in activation of macrophages from both compartments.

In conclusion, we have demonstrated the biological activity of a new form of immunomodulation, p.o. administration of liposome-encapsulated macrophage activators. This methodology resulted in systemic activation of tissue macrophages associated with the release of cytokines and the inhibition of experimental lung metastasis of murine renal cell carcinoma.

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