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In situ activation of mouse macrophages and therapy of spontaneous renal cell cancer metastasis by liposomes containing the lipopeptide CGP 31362

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Summary. We determined whether the intravenous administration of multilamellar vesicle liposomes (MLV) containing a lipopeptide analogue of a fragment from the cell wall of gram-negative bacteria (CGP 31362) can render BALB/c mouse alveolar macrophages tumoricidal in situ and reduce the incidence of spontaneous lung metastasis of syngeneic renal carcinoma (RENCA) cells. Alveolar macrophages (a) incubated in vitro with MLV containing CGP 31362 (MLV-31362) and (b) harvested from mice injected i.v. with MLV-31362 were rendered cytotoxic against the RENCA cells. Maximum cytotoxic activity of the macrophages was induced by injecting 5 μmol MLV consisting of 250 mg phospholipids and 0.5 mg CGP 31362. The single i.v. injection of 5 μmol MLV-31362 produced activation of macrophages that lasted for up to 4 days. Repeated i.v. injections of MLV-31362 produced a continuous antitumor activity in alveolar macrophages. To study the lipopeptide's effects on metastasis, we injected the left kidneys of BALB/c mice with RENCA cells. The kidney with growing tumor was resected 10 days later and, after a further 2 days, groups of mice were injected i.v. with MLV-31362 or with MLV-HBSS (twice weekly for 3 weeks). Treatment with MLV-31362 significantly decreased the median number of spontaneous lung metastases. These data demonstrate that the systemic administration of MLV-31362 can activate murine lung macrophages in situ and reduce the incidence of spontaneous RENCA lung metastases.

Key words: Mouse macrophages – Renal cell cancer – Metastasis – Liposomes – CGP 31362

Introduction

Efficient activation of macrophages in situ can be achieved by the encapsulation of immunomodulators within phospholipid liposomes [6, 7, 35]. Intravenously administered liposomes of appropriate phospholipid composition are cleared from the circulation by phagocytic cells [6, 7, 35]. The endocytosis of liposomes containing immunomodulators such as muramyl dipeptide (and various analogues or lymphokines such as interferon γ) renders macrophages cytotoxic in situ [4, 9, 13, 23]. The multiple administration of liposomes containing specific immunomodulators has been shown to eradicate cancer metastases in several murine tumor systems [2, 8, 29, 30, 39] and in dogs with lung metastases of osteogenic sarcoma [22]. Similarly, liposomes containing the lipophilic muramyl tripeptide phosphatidylethanolamine (PtdEtn) have been shown to activate tumoricidal properties in blood monocytes of cancer patients [12, 17, 20, 25, 37, 38]. Moreover, the results of phase I clinical trials indicate that at the minimum dose sufficient to produce desirable biological effects, these liposomes are well tolerated [3, 20, 25, 40].

N-Hexadecanoyl-*S*-[2(*R*)3-didodecanoyloxypropyl]-L-cysteinyl-L-alanyl-D-isoglutaminyl-glycyl-taurine sodium salt (CGP 31362) is a newly synthesized lipopeptide analogue of a fragment of lipoprotein from the outer wall of gram-negative bacteria. Its molecular mass is 1184 Da. It is not water-soluble but can be finely dispersed by sonication. It has been shown to induce potent activation of human blood monocytes at a lower concentration than muramyl tripeptide PtdEtn [14]. Monocytes treated with CGP 31362 release tumor necrosis factor (TNF), interleukin-1 (IL-1), and prostaglandin E_2, whereas those incubated with muramyl tripeptide PtdEtn release only TNF [14]. Moreover, we recently found that the encapsulation of CGP 31362 into phospholipid liposomes induced potent activation of murine peritoneal and bone marrow macrophages and human blood monocytes under in vitro conditions [27]. The purpose of this study was to examine whether the i.v. administration of liposomes containing CGP 31362 can render mouse alveolar macrophages cytotoxic in situ and thereby inhibit the growth of spontaneous lung metastases of renal carcinoma cells produced by local tumors in the kidney of syngeneic mice.

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Materials and methods

Animals. Specific pathogen-free mice of the inbred BALB/c strain, 6–8 weeks old, were purchased from the Animal Production Area, NCI-Frederick Cancer Research Facility (Frederick, Md). Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

Reagents. Minimum essential medium (MEM) and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, Md.). Hank's balanced salt solution (HBSS) was purchased from Mediatech (Herndon, Va.). CGP 31 362 was the gift of Ciba-Geigy, Ltd. (Basel, Switzerland). 1′,2′-Dioleoyl-*sn*-glycero-3-phospho-L-serine monosodium salt (Ole_2GroP-Ser) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PamOleGro*P*Cho) were synthesized at Ciba-Geigy [42]. All reagents contained less than 0.125 ng/ml endotoxins as detected by the *Limulus* amebocyte lysate assay (Associates of Cape Cod Inc., Woods Hole, Mass.)

Preparation of liposomes. PamOleGro*P*Cho at 175 mg and $Ole_2GroPSer$ at 75 mg with different concentrations (0–1 mg) of CGP 31 362 were dissolved under nitrogen in *n*-butanol at 50° C. The clear solution was sterile-filtered through a Gelman-TF-200 (0.2-μm filter). Aliquots (1 ml containing phospholipids with or without CGP 31 362) were lyophilized in standard vials (15 ml) using a Lyovac GT 4 lyophilizer. The resulting lyophilisates were sealed and stored under argon [33]. Multilamellar vesicle liposomes (MLV) were prepared at room temperature from the lyophilisate by the addition of 2.5 ml suspension medium (0.2 mg disodium salt of EDTA, 0.2 mg potassium chloride, 8.0 mg sodium chloride, 0.2 mg monobasic potassium phosphate, 1.2 mg dibasic sodium phosphate/ml water for injection) followed by hydration for 1 min and vigorous shaking for 2 min using a Vortex shaker at a high setting.

Tumor cells and culture conditions. The murine renal cell carcinoma line RENCA [24] was obtained from Dr. Robert Wiltrout, NCI-Frederick Cancer Research Facility [33], and maintained as a monolayer culture in MEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, 2 mM L-glutamine, 2 × vitamin solution, penicillin, and streptomycin (designated as CMEM) at 37° C in a humidified atmosphere containing 5% CO_2 and air. The cells were free of mycoplasma and pathogenic murine viruses.

Isolation and culture of mouse alveolar macrophages (AM). AM were harvested by a tracheobronchial lavage method as described previously [4, 8, 36]. Briefly, mice were anesthetized by i.p. injection of 0.15 ml sodium pentobarbital (10 mg/ml) and washed with alcohol. To minimize postmortem pulmonary edema and reduce trapped blood in the lung, we exsanguinated the mice by severing the left renal artery. After the chest was opened, a blunt needle (20 G) was inserted into the trachea and stabilized with surgical thread. The lungs were lavaged repeatedly with 0.8 ml HBSS prewarmed at 37° C until 10 ml total volume was delivered. This method yielded $(1-3) \times 10^5$ AM/mouse. The lavaged AM suspension was washed with HBSS and resuspended in serum-free MEM. The AM were plated at the density of $1 \times 10^5/38$ mm^2 well of microtest III plates (Falcon Plastics, Oxnard, Calif.) and allowed to adhere for 1.5 h at 37° C. Then, nonadherent cells were removed by washing with medium. The purity of the adherent AM was >95% as determined by morphology and phagocytosis of carbon particles [4, 8].

In vivo and in vitro activation of AM. For in vivo activation of AM, mice were given an i.v. injection of MLV containing either CGP 31 362 or HBSS in a volume of 0.1 ml HBSS/mouse. AM were harvested by lavage 24 h after the injection and plated as described above. The adherent cells were >95% AM.

For in vitro activation, AM harvested from normal mice were incubated with different concentrations of MLV containing CGP 31 362 or HBSS for 18 h. At that time, the cultures were thoroughly washed with medium before the addition of target cells.

In vitro assay of AM-mediated cytotoxicity. AM-mediated cytotoxicity was assessed by measuring the release of radioactivity from DNA of target cells as described previously [31, 32]. Briefly, RENCA target cells in their exponential growth phase were incubated for 24 h in supplemented MEM (CMEM) containing 0.5 μCi/ml [^{125}I]iododeoxyuridine, ([^{125}I]IdUrd; specific activity 2200 μCi/mmol, New England Nuclear, Boston, Mass.). The cells were washed twice with HBSS to remove unbound radioisotope and then harvested by a brief trypsinization (0.25% trypsin and 0.02% EDTA), washed, and resuspended in CMEM; 1×10^4 viable cells were plated into wells containing AM to achieve a population density of 2500 macrophages and 250 tumor cells/mm^2. At this population density, normal (untreated) macrophages are not cytotoxic to neoplastic cells [31, 32]. After a 72-h incubation, the cultures were washed twice with HBSS, and adherent viable cells were lysed with 0.1 ml 0.1 M NaOH. The lysates were absorbed into cotton swabs, and radioactivity was monitored in a gamma counter. Maximal in vitro macrophage-mediated cytotoxicity in this assay is obtained after 3 days of incubation with target cells, and macrophages do not reincorporate [^{125}I]IdUrd released from dead target cells [31, 32]. The cytotoxic activity of the macrophages was calculated as follows:

$$\text{Cytotoxicity } (\%) = [A-B]/A \times 100,$$

where A = radioactivity (cpm) in cultures of control AM and target cells, and B = radioactivity (cpm) in cultures of test AM and target cells.

Inhibition of spontaneous metastasis by intravenous injection of MLV containing CGP 31 362. RENCA cells harvested from adherent cultures (as described above) were washed twice with HBSS. The viability was >95% as assessed by trypan blue dye exclusion, and 1×10^4 single cells in 0.05 ml HBSS were injected into the subcapsule of the left kidney of syngeneic BALB/c mice. The kidney with growing tumor was resected 10 days later. In this tumor system, spontaneous lung metastases are established by the time the kidney with local tumor is removed. Systemic treatment with MLV began I day after nephrectomy. Each treatment consisted of 5 μmol MLV containing either HBSS (placebo) or 0.5 mg CGP 31 362 (in 250 mg phospholipid). The inoculum volume was 0.2 ml HBSS. The treatments were repeated twice weekly for 3 weeks (total of six i.v. injections). The mice were killed 28 days after tumor inoculation and necropsied. The lungs were removed, rinsed in water, and fixed in Bouin's solution for 24 h prior to fixation in 10% buffered formalin. The number of spontaneous metastases was determined with a dissecting microscope.

Statistical analysis. Experimental results were analyzed for their statistical significance by the two-tailed Student's *t*-test or the two-tailed Mann-Whitney *U*-test.

Results

In vitro activation of AM

In the first set of experiments, we examined whether the in vitro incubation of AM with MLV-31 362 can generate cytotoxic activity against syngeneic RENCA cells. AM harvested from normal BALB/c mice were incubated in medium or medium containing different concentrations of MLV (3–50 nmol/well) containing different quantities of CGP 31 362 (0, 0.25, 0.5, or 1 mg/250 mg phospholipids). The specific percentage cytotoxicity mediated by the AM was calculated by comparison with control AM (medium-incubated). The results of one representative experiment of three shown in Fig. 1 demonstrate that MLV-31 362 rendered AM cytotoxic in vitro after 18 h incubation with all MLV preparations (P <0.001), even 3 nmol/well. A plateau was reached by 12.5 nmol/well. The highest level of cytotoxicity was observed in cultures treated with

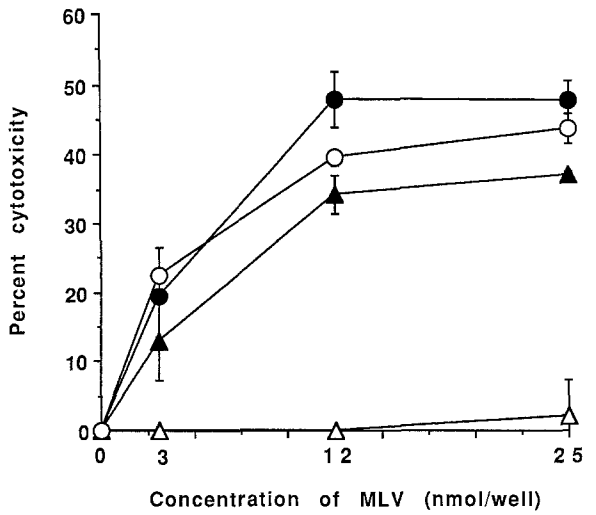

Fig. 1. In vitro activation of tumoricidal properties in murine alveolar macrophages (AM). AM harvested from normal mice were plated at a density of 1×10^5/38 mm^2 wells and treated for 18 h with 0, 3, 12.5, or 25 nmol/well of multilamellar vesicle liposomes (*MLV*) containing 0 mg (△), 0.25 mg (▲), 0.5 mg (●), or 1 mg (○) CGP 31362 (in 250 mg phospholipids). After this incubation, the AM cultures were thoroughly washed and 1×10^4 viable [^{125}I]IdUrd-labeled renal carcinoma (RENCA) cells were added. AM-mediated tumor lysis was determined 72 h later. Values are mean ± SD of triplicate cultures. This is one representative experiment of three

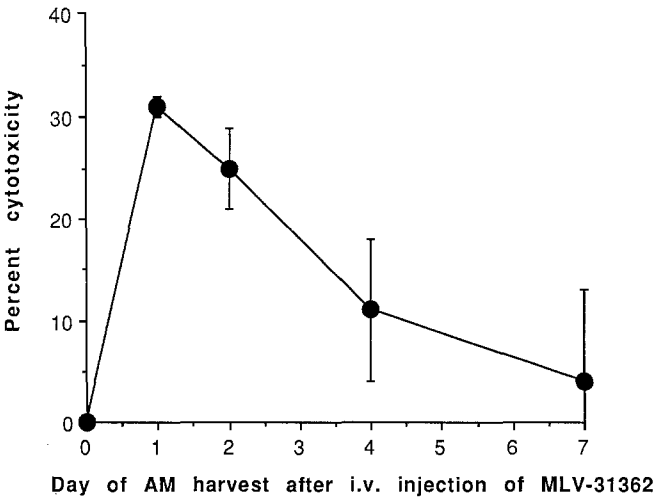

Fig. 2. The relationship of CGP 31362 concentration in MLV on the in vivo induction of cytotoxic activity in AM. Five mice per group were injected with 5 μmol MLV containing 0 mg, 0.125 mg, 0.25 mg, 0.5 mg, and 1 mg CGP 31362 (in 250 mg phospholipids). AM were harvested 24 h later and their tumoricidal activity was assayed in vitro against [^{125}I]IdUrd-labeled RENCA cells. The results are mean ± SD of triplicate cultures. This is one representative experiment of three

12.5 nmol/well MLV containing 0.5 mg CGP 31362 (per 250 mg phospholipids). MLV containing HBSS (placebo) did not produce any significant tumoricidal activity in the AM.

In vivo activation of AM

In the next set of experiments, we examined whether the i.v. administration of MLV-31362 can produce in vivo activation of AM. Mice were injected i.v. with 5 μmol MLV containing different quantities of CGP 31362 (0, 0.125, 0.25, 0.5, or 1.0 mg/250 mg phospholipids). AM were harvested 24 h later, and then cytotoxic properties were measured ex vivo. The results of one representative experiment of three are shown in Fig. 2. The single i.v. injection of 5 μmol MLV containing different quantities of CGP 31362 produced in vivo activation of AM in a dose-dependent manner. MLV containing 0.5 mg CGP 31362 in 250 mg phospholipids were most effective in inducing activation of AM. Control preparations of MLV containing HBSS (placebo) did not produce significant activation of AM. To examine further the relationship between the dose of MLV phospholipid and the concentration of CGP 31362 in their effect on the in vivo activation of AM, we injected mice i.v. with different amounts (0, 2.5, 5, or 10 μmol) of MLV containing different concentrations of CGP 31362 (0, 0.25, 0.5, 1.0 mg in 250 mg phospholipids). AM were harvested 24 h after the i.v. injection and cytotoxicity was assessed in vitro. The results are

Table 1. In situ activation of tumoricidal properties in murine alveolar macrophages (AM) following i.v. injection of multilamellar vesicle liposomes (MLV) containing CGP 31362[a]

Dose of MLV (μmol/mouse)	Concentration of CGP 31362 (in 250 mg MLV phospholipids) (cpm)			
	0 mg	0.25 mg	0.5 mg	1 mg
0	1385 ± 53[b]	975 ± 45	141 ± 10	1338 ± 30
2.5	ND[c]	855 ± 0 (12.3)[d]	984 ± 120 (30.3)[d]	834 ± 93 (37.7)[d]
5	1325 ± 57 (4.7)	738 ± 2 (24.3)[d]	720 ± 31 (49.0)[d]	888 ± 33 (33.6)[d]
10	ND	585 ± 59 (40.0)[d]	1071 ± 43 (24.1)[d]	1294 ± 32 (4.0)
Tumor cells alone	1515 ± 20	1098 ± 87	1946 ± 3	1722 ± 78

[a] Mice (n = 5) were injected i.v. with the indicated amount of MLV containing different concentrations of CGP 31362. AM were harvested 24 h after i.v. injection of liposomes and 1×10^5 AM were plated into 38-mm^2 wells for 1.5 h; 1×10^4 [^{125}I]IdUrd-labeled renal carcinoma (RENCA) cells were then plated into the wells with adherent AM or alone as an additional control. AM-mediated tumor cell lysis was determined after 72 h of cocultivation. This is one representative experiment of three

[b] Mean radioactivity ± SD of viable cells in triplicate cultures

[c] Not done

[d] The number in parenthesis is the percentage AM-mediated cytotoxicity calculated by comparison with AM from control mice; $P < 0.001$

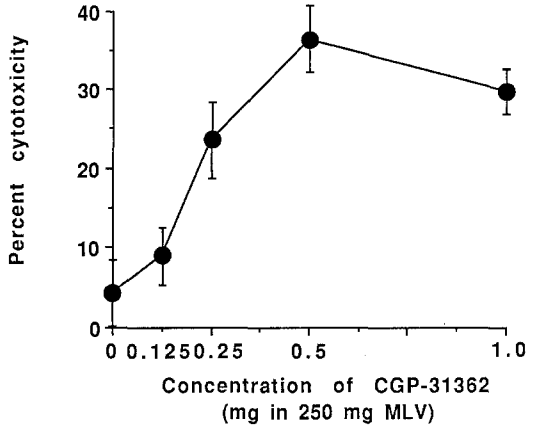

Fig. 3. Duration of tumoricidal activity in AM activated in situ with MLV-31 362. Five mice per group were injected with a single dose of 5 µmol MLV containing 0.5 mg CGP 31 362 (in 250 mg phospholipids). AM were harvested on the indicated days after the i.v. injection and 1×10^5 AM were plated into 38-mm^2 wells for 1.5 h. The cultures were washed and 1×10^4 [^{125}I]IdUrd-labeled RENCA cells were added. AM-mediated cytotoxicity was determined 72 h later. The results are mean ± SD of triplicate cultures. This is one representative experiment of two

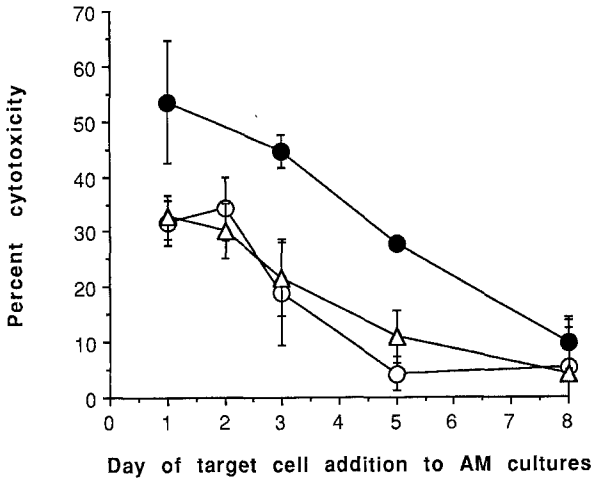

Fig. 4. Maintenance of tumoricidal properties in AM activated in situ with MLV-31 362. Mice were injected with a single dose of 5 µmol MLV containing 0.25 mg (△), 0.5 mg (●), or 1 mg (○) of CGP 31 362 (in 250 mg phospholipids). AM were harvested 24 h after i.v. injection (day 1) and cultured in vitro in medium for the indicated intervals. [^{125}I]IdUrd-labeled RENCA cells were added to different AM cultures at the indicated intervals. Tumor lysis was determined after 72 h of cocultivation. The results are mean ± SD of triplicate cultures. This is one representative experiment of two

shown in Table 1. The optimal activation of cytotoxic properties in AM depended upon both the amount of MLV injected and the concentration of CGP 31 362 incorporated within. A linear relationship was found. Optimal activation for MLV containing 0.25 mg CGP 31 362 was achieved with i.v. injection of 10 µmol MLV. For MLV containing 0.5 mg CGP 31 362 it was 5 µmol, and for MLV containing 1 mg CGP 31 362 it was 2.5 µmol MLV. As long as more than 2.5 µmol MLV was injected i.v., the degree of AM activation in vivo was clearly dependent on the dose of MLV and the concentration of CGP 31 362 in the MLV.

Table 2. Maximal in situ activation of AM[a]

Number of i.v. injections	AM tumoricidal activity (cpm)
0	902 ± 31[b]
1	576 ± 54 (36.2)[c]
2	394 ± 32 (56.3)[c]
3	484 ± 30 (46.3)[c]
Tumor cells alone	1043 ± 55

[a] Five mice per group were injected i.v. with 5 µmol MLV containing 0.5 mg CGP 31 362 (in 250 mg phospholipids). The injections were performed one to three times every 3 days. AM were harvested 24 h after the final injection and plated into culture wells. AM-mediated tumor lysis was determined in vitro against [^{125}I]IdUrd-labeled RENCA cells. This is one experiment of two

[b] Mean radioactivity ± SD of triplicate cultures

[c] The number in parenthesis is the percentage AM-mediated cytotoxicity calculated by comparison to AM from untreated mice; $P < 0.001$

The highest level of AM activation was produced by injecting 5 µmol MLV containing 0.5 mg CGP 31 362.

We next determined the duration of AM tumoricidal activity subsequent to the i.v. injection of a single dose of MLV 31 362 (5 µmol MLV with 0.5 mg CGP 31 362 in 250 mg phospholipid). In the first set of experiments, we harvested AM 1, 2, 4, or 7 days after the injection of MLV and assessed their antitumor activity in vitro. As the results in Fig. 3 demonstrate, 1 day after the i.v. injection, the AM produced 31% lysis of the RENCA cells, which activity declined to 12% by day 4.

In the second set of experiments, we injected mice with a single dose of 5 µmol MLV containing 0.25 mg, 0.5 mg, or 1.0 mg CGP 31 362/250 mg phospholipids. AM were harvested 1 day after the i.v. injection and cultured in vitro for an additional 1, 2, 4, or 7 days. Viability of AM even at day 8 exceeded 97%. RENCA cells were added to AM at different times, and cell lysis was assayed. As shown in Fig. 4, AM harvested 1 day after the i.v. injection of 5 µmol MLV containing 0.5 mg CGP 31 362/250 mg phospholipids produced the highest level of cytotoxic activity, which remained at significant levels (30%) for 4 days.

To investigate the effect of multiple i.v. administrations of MLV-31 362 on maintenance of cytotoxic activity of AM, we injected mice once, twice, or three times with 5 µmol MLV (containing 0.5 mg CGP 31 362/250 mg phospholipids) every 3 days. AM harvested 24 h after one, two, or three injections were assessed for antitumor activity. The results shown in Table 2 demonstrate that repeated i.v. injections of MLV-31 362 maintained the tumoricidal activity of AM.

Inhibition of spontaneous metastases by the i.v. administration of MLV-31 362

In the final set of experiments, we examined whether repeated i.v. injections of MLV-31 362 can influence the development and growth of spontaneous RENCA lung metastases. We injected RENCA cells into the kidney subcapsule, 10 days later resected this kidney, and 2 days later

Table 3. Inhibition of spontaneous RENCA lung metastasis by i. v. injection of MLV-31 362[a]

Expt.	Treatment of mice	Number of lung metastases		Regional recurrence
		Median	Range	
1	HBSS	42	12–150	6/ 8
	MLV-HBSS	49	25–150	6/ 6
	MLV-31 362	21*	0– 55	0/ 8**
2	HBSS	48	16–150	7/10
	MLV-31 362	14***	4– 44	2/10**

[a] Mice ($n = 10$) were injected with 1×10^4 viable RENCA cells into the left kidney subcapsule. This kidney was removed 10 days later and MLV treatment began 2 days thereafter: 5 μmol MLV containing either Hanks' balanced salt solution (HBSS; placebo) or 0.5 mg CGP 31 362 (in 250 mg phospholipids) suspended in 0.1 ml HBSS was injected i. v. twice weekly for 3 weeks. Mice were killed 28 days after tumor inoculation and the number of metastases was determined under a dissecting microscope

* $P < 0.02$
** $P < 0.01$
*** $P < 0.005$

began MLV treatment. The mice were injected twice weekly for 3 weeks. Each i. v. dose consisted of 5 μmol MLV with 0.5 mg CGP 31 362 or 5 μmol MLV-HBSS. The mice were killed when controls became moribund. The incidence of spontaneous lung metastasis is summarized in Table 3. The median number of spontaneous lung metastases in control mice (HBSS-treated) was 42 (experiment 1) and 48 (experiment 2). Treatment with MLV-HBSS did not change the outcome. In contrast, treatment with MLV-31 362 reduced the median number of RENCA lung metastases to 21 (experiment 1, $P < 0.02$) and 14 (experiment 2, $P < 0.005$). In this tumor model, the resection of the injected kidney does not prevent the subsequent growth of RENCA in the peritoneum (local-regional recurrence); however, systemic therapy with MLV-31 362 was effective in reducing regional recurrences as well (Table 3).

Discussion

The activation of macrophages by liposomes containing immunomodulators occurs subsequent to phagocytic uptake of the liposomes and the interaction of encapsulated immunomodulating molecules with intracellular targets [11, 15]. Activated macrophages can produce more than 100 distinct molecules [26]. Some of these diffusible mediators affect tumor cells and include oxygen metabolites [26], TNF [18], IL-1 [18, 21, 28], proteases [1], and reactive nitrogen intermediates [16]. Since the interaction of macrophages with different agents induces the production and release of a large number of products, we reasoned that different activation signals could lead to the release of different molecules, and therefore recently compared the efficacy of liposomes containing CGP 31 362 or CGP 19 835 for activating mouse macrophages and human monocytes under in vitro conditions [14, 41]. MLV-19 835 activated monocytes only in the presence of interferon-γ, whereas activation with MLV-31 362 was interferon-independent. Activation of both mouse macrophages and human blood monocytes by MLV-31 362 occurred at a lower liposomal concentration than that by MLV-19 835, and monocytes incubated with MLV-31 362-released prostaglandin E_2, TNF, and IL-1 activities whereas monocytes treated with MLV-19 835 released only TNF activity [41]. These data suggested that liposomes containing the synthetic lipopeptide CGP 31 362 are superior to liposomes containing MTP-PE for systemic activation of macrophages and prompted us to study these liposomes for in situ activation of macrophages.

Our results demonstrate that the i. v. injection of liposomes consisting of phosphatidylcholine (PtdCho) and phosphatidylserine (PtdSer) (7:3 molar ratio) with entrapped CGP 31 362 can render mouse AM tumoricidal in situ. Maximal activation of AM was produced after the i. v. injection of 5 μmol MLV containing 0.5 mg CGP 31 362 in 250 mg phospholipids. Subsequent to a single i. v. injection, the activated AM maintained their cytotoxic activity up to 4 days. Since therapy of lung metastases requires a long duration of macrophage activity [5], we examined whether continuous cytotoxic activity of AM could be maintained by repeated i. v. injections of MLV-31 362; our present data show that this can be achieved by injecting MLV-31 362 every 3 days. On the basis of these parameters, we examined whether repeated i. v. injections of MLV-31 362 could inhibit the growth of spontaneous RENCA lung metastases. We observed a direct correlation between in situ activation of AM and inhibition of RENCA lung metastases.

Maximal in vivo activation of AM by MLV containing CGP 31 362 was influenced by both the amount (dose) of MLV and the proportion (concentration) of CGP 31 362. Optimal AM activation was found subsequent to the i. v. injection of 5 μmol MLV containing 0.5 mg CGP 31 362 (per 250 mg phospholipids). Exceeding 0.5 mg CGP 31 362 in the MLV was not productive. AM from mice injected with a high dose of MLV containing 1.0 mg CGP 31 362 were less cytotoxic than AM from mice injected with lower doses.

In the present study, CGP 31 362 was incorporated into PtdCho and PtdSer MLV (7:3 molar ratio). We chose this phospholipid composition because these MLV rapidly bind to macrophages and efficiently deliver immunomodulators to these cells [27]. Because of its lipophilic nature, CGP 31 362 is directly inserted into the phospholipid bilayer of MLV; therefore, the in vivo distribution of CGP 31 362 is determined by their phospholipid composition [10]. Previous studies from our laboratory have shown that liposomes consisting of PtdCho and PtdSer (7:3 molar ratio) are cleared from the circulation by fixed and free phagocytic cells [6, 7, 35]. Most important, about 10% of i. v. injected PtdCho/PtdSer MLV can localize in the lung microvasculature [34]. Subsequently, the MLV are phagocytosed by blood monocytes that in turn migrate into the lung parenchyma to mature into lung macrophages and then AM [19]. Both the lung macrophages and AM can destroy metastatic tumor cells [5], and our present results show that AM activated in situ can lyse RENCA cells. Moreover, activation of lung macrophages by MLV-31 362 occurs in mice treated with antibodies to asialo-GM1, whose natural killer (NK) cells are suppressed (Utsugi et

al. manuscript in preparation). Although these data do not rule out the possibility that the eradication of RENCA metastases was due in part to effector cells such as NK cells, they do suggest that macrophages play a major role.

In summary, we demonstrate that the i.v. injection of MLV containing CGP 31362 can render mouse AM tumoricidal. Maximal activation of AM in situ was induced by the injection of 5 μmol MLV containing 0.5 mg CGP 31362 in 250 μmol phospholipids. Multiple i.v. injections of MLV containing CGP 31362 produced significant inhibition of spontaneous lung metastases of renal cell carcinoma growing in the kidney. The efficacy of MLV-31362 for therapy of other murine metastatic tumors is now under study.

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References

1. Adams DO, Kao K-J, Farb R, Pizzo SV (1980) Effector mechanisms of cytolytically activated macrophages: II. Secretion of a cytolytic factor by activated macrophages and its relationship to secreted neural proteases. J Immunol 124: 293
2. Brodt P, Blore J, Phillips NC, Munzer JS, Rioux JD (1989) Inhibition of murine hepatic tumor growth by liposomes containing a lipophilic muramyl dipeptide. Cancer Immunol Immunother 28: 54
3. Creaven PJ, Cowens JW, Brenner DE, Dadey BM, Han T, Huben R, Karakousis C, Frost H, LeSher D, Hanagan J, Andrejcio K, Cushman MK (1990) Initial clinical trial of the macrophage activator muramyl tripeptide-phosphatidylethanolamine encapsulated in liposomes in patients with advanced cancer. J Biol Response Mod 9: 492
4. Fidler IJ (1981) The in situ induction of tumoricidal activity in alveolar macrophages by liposomes containing muramyl dipeptide is a thymus-independent process. J Immunol 127: 1719
5. Fidler IJ (1985) Macrophages and metastasis – a biological approach to cancer therapy: presidential address. Cancer Res 45: 4714
6. Fidler IJ (1988) Targeting of immunomodulators to mononuclear phagocytes for therapy of cancer. Adv Drug Delivery Rev 2: 69
7. Fidler IJ, Raz A, Fogler WE, Kirsh R, Bugelski P, Poste G (1980) The design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. Cancer Res 40: 4460
8. Fidler IJ, Sone S, Fogler WE, Barnes ZL (1981) Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing muramyl dipeptide. Proc Natl Acad Sci USA 78: 1680
9. Fidler IJ, Sone S, Fogler WE, Smith D, Braun DG, Tarcsay L, Gisler RJ, Schroit AJ (1982) Efficacy of liposomes containing a lipophilic muramyl dipeptide derivative for activating the tumoricidal properties of alveolar macrophages in vivo. J Biol Response Mod 1: 43
10. Fidler IJ, Fogler WE, Tarcsay L, Schumann G, Braun DG, Schroit AJ (1983) Systemic activation of macrophages and treatment of cancer metastases by liposomes containing hydrophilic or lipophilic muramyl dipeptide. Immunopharmacol 2: 253
11. Fidler IJ, Fogler WE, Kleinerman ES, Saiki I (1985) Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human gamma interferon encapsulated in liposomes. J Immunol 135: 4289
12. Fidler IJ, Jessup JM, Fogler WE, Staerkel R, Mazumder A (1986) Activation of tumoricidal properties in peripheral blood monocytes of patients with colorectal carcinoma. Cancer Res 44: 994
13. Fidler IJ, Fan D, Ichinose Y (1989) Potent in situ activation of murine lung macrophages and therapy of melanoma metastases by systemic administration of liposomes containing muramyltripeptide phosphatidylethanolamine and interferon gamma. Invasion Metastasis 9: 75
14. Fidler IJ, Nii A, Utsugi T, Brown D, Bakouche O, Kleinerman ES (1990) Differential release of TNF-α, IL 1, and PGE_2 by human blood monocytes subsequent to interaction with different bacterial derived agents. Lymphokine Res 9: 449
15. Fogler WE, Fidler IJ (1986) The activation of tumoricidal properties in human blood monocytes by muramyl dipeptide requires specific intracellular interaction. J Immunol 136: 2311
16. Hibbs JB, Taintor RR, Vavrin Z (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrate. Science 235: 473
17. Hudson MM, Snyder JS, Jaffe N, Kleinerman ES (1988) In vitro and in vivo effect of Adriamycin therapy on monocyte activation by liposome-encapsulated immunomodulators. Cancer Res 48: 5256
18. Ichinose Y, Bakouche O, Tsao JY, Fidler IJ (1988) Tumor necrosis factor and IL-1 associated with plasma membranes of activated human monocytes lyse monokine-sensitive but not monokine-resistant tumor cells whereas viable activated monocytes lyse both. J Immunol 141: 512
19. Key ME, Talmadge JE, Fogler WE, Bucana CD, Fidler IJ (1982) Isolation of tumoricidal macrophages from lung melanoma metastases of mice treated systemically with liposomes containing a lipophilic derivative of muramyl dipeptide. J Natl Cancer Inst 69: 1189
20. Kleinerman ES, Murray JL, Snyder JS, Cunningham JE, Fidler IJ (1989) Activation of tumoricidal properties in monocytes from cancer patients following intravenous administration of liposomes containing muramyl tripeptide phosphatidylethanolamine. Cancer Res 49: 4665
21. Lachman LB, Dinarello CA, Llansa ND, Fidler IJ (1986) Natural and recombinant human interleukin 1-β is cytotoxic for human melanoma cells. J Immunol 136: 3098
22. MacEwen EG, Kurzman ID, Rosenthal RC, Smith BW, Manley PA, Roush JK, Howard PE (1989) Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. J Natl Cancer Inst 81: 935
23. Mehta K, Lopez-Berestein G, Hersh EM, Juliano RL (1982) Uptake of liposomes and liposome-encapsulated muramyl dipeptide by human peripheral blood monocytes. J Reticuloendothel Soc 32: 155
24. Murphy GP, Hrushesky WJ (1973) A murine renal cell carcinoma. J Natl Cancer Inst 50: 130
25. Murray JL, Kleinerman ES, Cunningham JE, Tatom JR, Andrejcio K, Lepe-Zuniga J, Lamki LM, Rosenblum MG, Frost H, Gutterman JU, Fidler IJ (1989) Phase I trial of liposomal muramyl tripeptide phosphatidylethanolamine [MTP-PE (CGP 19835A)] in cancer patients. J Clin Oncol 7: 915
26. Nathan CF, Silverstein SC, Brukner LH, Cohn ZA (1979) Extracellular cytolysis by activated macrophages and granulocytes: II. Hydrogen peroxide as a mediator of cytotoxicity. J Exp Med 149: 100
27. Nii A, Utsugi T, Fan D, Denkins Y, Pak C, Brown D, van Hoogevest P, Fidler IJ (1991) Development of liposomes containing a new lipopeptide CGP 31362 for efficient activation of tumoricidal properties in monocytes and macrophages. J Immunother (in press)
28. Onozaki K, Matsushima K, Aggarwal BB, Saito T, Oppenheim JJ (1985) Human interleukin-1 is a cytocidal factor for several tumor cell lines. J Immunol 135: 3862
29. Phillips NC, Tsao M (1989) Inhibition of experimental liver tumor growth in mice by liposomes containing a lipophilic muramyl dipeptide. Cancer Res 49: 936
30. Phillips NC, Mora ML, Chedid L, Lefrancier P, Bernard JM (1985) Activation of tumoricidal activity of experimental metastases by freeze-dried liposomes containing a new lipophilic muramyl dipeptide derivative. Cancer Res 45: 128
31. Raz A, Fogler WE, Fidler IJ (1979) The effect of experimental condition on the expression of in vitro mediated tumor cytotoxicity mediated by murine macrophages. Cancer Immunol Immunother 7: 157
32. Saiki I, Fidler IJ (1985) Synergistic activation by recombinant mouse interferon-γ and muramyl dipeptide of tumoricidal properties in mouse macrophages. J Immunol 135: 684

33. Salup RR, Herberman RB, Wiltrout RH (1985) Role of natural killer activity in the development of spontaneous metastasis in murine renal cancer. J Urol 134: 1236
34. Schroit AJ, Fidler IJ (1982) Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. Cancer Res 42: 161
35. Schroit AJ, Hart IR, Madsen J, Fidler IJ (1983) Selective delivery of drugs encapsulated in liposomes: natural targeting to macrophages involved in various disease states. J Biol Response Mod 2: 97
36. Sone S, Fidler IJ (1981) In vitro activation of tumoricidal properties in rat alveolar macrophages by synthetic muramyl dipeptide encapsulated in liposomes. Cell Immunol 57: 42
37. Sone S, Tandon P, Utsugi T, Ogawara M, Shimizu E, Nii A, Ogura T (1986) Synergism of recombinant interferon gamma with liposome-encapsulated muramyl tripeptide in activation of the tumoricidal properties of human monocytes. Int J Cancer 38: 495
38. Sone S, Utsugi T, Tandon P, Ogawara M (1986) A dried preparation of liposomes containing muramyl tripeptide phosphatidylethanolamine as a potent activator of human blood monocytes to the antitumor state. Cancer Immunol Immunother 22: 191
39. Talmadge JE, Lenz BF, Klabansky R, Simon R, Rigs C, Guo S, Oldham RK, Fidler IJ (1986) Therapy of autochthonous skin cancers in mice with intravenously injected liposomes containing muramyl tripeptide. Cancer Res 46: 1160
40. Urba WJ, Hatmann LC, Longo DL, Steis RG, Smith II JW, Kedar I, Creekmore S, Sznol M, Conlon K, Kopp WC, Huber C, Herold M, Alvord WG, Snow S, Clark JW (1990) Phase I and immunomodulatory study of a muramyl peptide, muramyl tripeptide phosphatidylethanolamine. Cancer Res 50: 2979
41. Utsugi T, Nii A, Fan D, Pak CC, Denkins Y, van Hoogevest P, Fidler IJ (1991) Comparative efficacy of liposomes containing synthetic bacterial cell wall analogues for tumoricidal activation of monocytes and macrophages. Cancer Immunol Immunother 33: 285–292
42. van Hoogevest P, Fankhauser P (1989) An industrial liposomal dosage form for muramyl-tripeptide-phosphatidylethanolamine (MTP-PE). In: Lopez-Berestein G, Fidler IJ (eds). Liposomes in the therapy of infectious diseases and cancer. Liss, New York, p 453