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Inhibition of Murine Hepatic tumor growth by liposomes containing a lipophilic muramyl dipeptide

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Summary. We have investigated the ability of liposomes containing a lipophilic muramyl dipeptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine glycerol dipalmitate (MDP-GDP) to activate Kupffer cell tumoricidal activity in situ and to inhibit the growth of experimental hepatic micrometastases of tumor cell line H-59, a liver-homing variant of the Lewis lung carcinoma. Liposomes prepared from distearoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DSPC/DMPG) and containing MDP-GDP (1 μmol and 2 μg, respectively) were efficiently taken up by the liver after i.v. administration. A single i.v. injection of DSPC/DMPG liposomes containing MDP-GDP was capable of inducing Kupffer cell tumoricidal activity against H-59 tumor cells as measured in vitro. Control liposomes or 100 μg free MDP were ineffective in inducing Kupffer cell tumoricidal activity in situ. Two treatment regimens were evaluated in vivo: firstly, C57BL/6 mice were injected with tumor cell line H-59 and subsequently treated with multiple injections of liposomal MDP-GDP. Secondly, treatment with liposomal MDP-GDP was initiated prior to tumor cell injection and continued after tumor cell injection. The ability of liposomes containing MDP-GDP to reduce the number of hepatic micrometastases using the first protocol was related to the tumor cell inoculum, significant inhibition being observed at lower liver tumor burdens (< 25 tumor nodules). Pretreatment of the mice prior to tumor cell challenge followed by treatment afterwards greatly enhanced the efficacy of liposomal MDP-GDP and brought about a highly significant inhibition of the growth of experimental metastases even at high liver tumor burdens (> 50 nodules).

Introduction

The fixed macrophage of the liver, the Kupffer cell, is an attractive target for the nonspecific immunotherapy of primary and metastatic liver cancer. It is present in large numbers throughout the liver, is continually being replaced by incoming monocytes [5], and is in close proximity to growing tumors. A number of studies have demonstrated the tumoricidal or tumoristatic properties of Kupffer cells [4, 7, 20]. Kupffer cell tumoricidal activity has been induced in vitro with immunomodulators such as lipopolysaccharide or macrophage activating factor [28], and in situ with *Mycobacterium bovis* or relatively high doses of a lipophilic muramyl tripeptide, MTP-PE [27].

Liposomes, small phospholipid vesicles, are able to incorporate and retain immunopharmacological agents. Liposomes are avidly taken up by cells of the reticuloendothelial system after i.v. administration. The localization of a substantial proportion of these liposomes within the fixed macrophage population of the liver, the Kupffer cell [19], provides the rational for using liposomes to target agents capable of activating Kupffer cell tumoricidal activity. In addition, Kupffer cell tumoricidal activity can be induced by liposome-encapsulated C-reactive protein or crude lymphokine [25].

There is some evidence that the antitumor potential of Kupffer cells can be utilized in a therapeutic manner. The ability of glucan, a β1, 3-linked glucopyranose, to inhibit the growth of experimental hepatic metastases of tumor M5076 [26] has been shown to correlate with its ability to induce Kupffer cell cytolytic activity after i.v. administration [21].

N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP), the minimal structural unit of bacterial cell wall peptidoglycan which possesses immunoadjuvant activity [8], is a potent activator of macrophage cytotoxic [24] and cytostatic activities [14]. Liposomal incorporation of MDP results in enhanced activity [15, 22], but its efficiency may be curtailed by low efficiency of entrapment and poor retention [15]. MDP, both in the free form and in lipsosmes, has also been shown to be a potent activator of Kupffer cell tumoricidal activity in vitro, liposomal incorporation resulting in an increase in the potency of MDP [6]. To date, no information is available on the relative efficacy of liposomal MDP preparations in inducing Kupffer cell tumoricidal activity in situ.

We have previously shown that lipophilic derivatives of MDP are efficiently incorporated and retained within liposomes, and are considerably more potent than free MDP or liposomal MDP in inducing murine alveolar macrophage tumoricidal activity in vitro [15, 16] or in situ [15, 17].

One of the factors which has limited the number of studies on the treatment of hepatic cancer is the paucity of experimental models in which hepatic metastases can be

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demonstrated in the absence of surgical manipulation. In this study we have utilized a liver-homing subline of the Lewis lung carcinoma, H-59, to investigate the potential of liposomes containing a lipophilic muramyl dipeptide, MDP-glycerol dipalmitate GDP), in preventing the growth of experimental hepatic micrometastases. Our results indicate that such liposome preparations are effective in preventing the growth of such tumors with a concomitant activation of Kupffer cell tumoricidal activity.

Materials and methods

Animals. Female C57BL/6 mice, 8–12 weeks of age, were supplied by Charles River Ltd., St. Constant, Quebec, Canada. Animals were regularly screened for the presence of common laboratory pathogens including murine hepatitis virus.

Tumor. Tumor cell line H-59, a highly metastatic subline of the Lewis lung carcinoma, was established from an hepatic metastasis of the parent line [3]. The tumor was maintained in vivo by s.c. implantation of hepatic nodules isolated from tumor-bearing animals. Single cell suspensions were obtained by mincing s.c. tumors and dispersing in 0.02% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS)-EDTA as previously described [3]. Tumor cells were routinely cultured in vitro in supplemented RPMI 1640 medium for 1 week in order to remove tumor-infiltrating host cells. Prior to inoculation single cell suspensions were prepared by incubation in PBS-EDTA, washing in Hanks' balanced sait solution (HBSS), and the cell suspensions adjusted to give the required number of viable tumor cells.

Reagents. The MDP and MDP-GDP were supplied by Dr. L. Chedid, University of South Florida Medical Center, Tampa, Fla. Distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylglycerol (DMPG) and *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dipalmitoylphosphatidylethanolamine (NBD-PE) were supplied by Avanti Polar Lipids, Birmingham, Ala. The phospholipids were kept in chloroform solution under N_2 at $-80°$ C until liposome preparation. Phospholipid purity was verified at weekly intervals by thin-layer chromatography on silica gel plates. All reagents were verified to be endotoxin free (Limulus amebocyte assay), and all glassware was treated at 180° C for 3 h to inactivate endotoxins.

Tissue distribution studies of i.v. injected liposomes. Liposomes (1 μmol) containing MDP-GDP and spiked with 0.5 μCi ^{14}C-DSPC (sp. act. 115 mCi/mmol, Amersham International, Mississauga, Ontario, Canada) were injected via a lateral tail vein into groups of 5 mice. The mice were sacrificed after 4 and 24 h, and the livers, spleens, kidneys, and lungs removed and weighed. An aliquot of heparinized blood was obtained immediately prior to sacrifice from the orbital plexus. Samples of the tissues were digested (Protosol; NEN, Montreal, Canada), and the radioactivity determined by liquid scintillation counting. All counts were corrected for quenching before calculation of the tissue distribution.

Liposome preparation. Multilamellar liposomes were prepared as previously described [15]. Briefly, DSPC and DMPG in the molar ratio 10:1 were admixed with MDP-GDP in chloroform in round-bottomed glass flasks and rotary evaporated to dryness under vacuum. The dried lipid film was hydrated with PBS at 20° C for 5 min, then vortexed at 60° C to form liposomes. Control liposomes were prepared by omitting the MDP-GDP. The MDP-GDP incorporation into the liposomal carriers was determined by analysis of *N*-acetylamino sugar as previously described [17]. The incorporation was routinely found to be 100% efficient. The NBD-PE was incorporated into the liposomes by adding the appropriate quantity in chloroform solution to the phospholipids prior to rotary evaporation (5 μg/μmol phospholipid).

In situ activation of Kupffer cells by liposomes containing MDP-GDP. Liposomes were administered to groups of 3 mice (1 μmol phospholipid or 1 μmol phospholipid containing 2 μg MDP-GDP) 24 h prior to harvesting of Kupffer cells.

Kupffer cell isolation. The mice were placed in a laminar-flow hood, anesthetized with ether, and exsanguinated. The liver was exposed, and perfused with 10 ml Ca^{2+}- and Mg^{2+}-free HBSS at 37° C followed by 10 ml Ca^{2+}- and Mg^{2+}-free HBSS containing 0.5 mg/ml collagenase and 0.01 mg/ml DNase II (Boehringer Mannheim, Dorval, Quebec, Canada). The livers from each treatment group were excised and combined, passed through a 60 μm stainless steel screen, and incubated with Ca^{2+}- and Mg^{2+}-free HBSS containing 0.5 mg/ml collagenase and 0.01 mg/ml DNase II (10 ml/gm wet weight tissue) for 45 min at 37° C in a shaking water bath. After elimination of residual hepatocytes by a series of low speed centrifugations (3×30 *g* for 2 min), the nonparenchymal cell fraction containing the Kupffer cells was admixed with 30% Metrizamide in HBSS (2.5 ml cell suspension plus 3.5 ml Metrizamide) and centrifuged at 2000 *g* for 15 min at 4° C [27]. The band containing the nonparenchymal cells was washed 3 times with HBSS to remove Metrizamide, and the final cell pellet was suspended in minimum essential medium–fetal calf serum (MEM-FCS). The nonparenchymal cells (4×10^5 cells containing 2×10^5 Kupffer cells) were plated in 96-well tissue culture microplates. Nonadherent cells were removed by gentle washing with MEM-FCS after 24 h incubation at 37° C/5% CO_2. The recovery of Kupffer cells was determined in separate experiments by the injection of India ink or of liposomes (1 μmol) containing the fluorescent probe NBD-PE 24 h before Kupffer cell preparation.

Tumoricidal assay. The H-59 tumor cells in monolayer culture were labeled with ^{3}H-methylthymidine (NEN; sp. act. 1 mCi/mmol) for 18 h at a concentration of 0.4 μCi/ml. After washing to remove unincorporated radiolabel, the cells were trypsinized (< 0.5 min), resuspended in MEM-FCS, and 5×10^3 viable tumor cells added to the Kupffer cell preparations to give a Kupffer to tumor cell ratio of 20:1. After incubation for 72 h, nonadherent dead cells were eliminated by washing each well 3 times with warm HBSS, after which the contents were lysed by the addition of 200 μl 0.5 *M* NaOH. The remaining radioactivity in the lysate and 2 further washes with NaOH were admixed with 5.6 ml Aquafluor (NEN) and the radioactivity determined in a liquid scintillation counter. All counts were corrected for quenching before determination of cytotoxic activity.

The cytotoxicity was determined using the equation:

$$\frac{[\text{cpm H-59 alone}] - [\text{cpm H-59} + \text{Kupffer cells}]}{[\text{cpm H-59 alone}]} \times 100$$

Treatment of H-59 liver tumors. For the therapeutic regimen, mice were injected via a lateral tail rein with $2-5 \times 10^5$ viable tumor cells. Immunoadjuvant therapy consisted of 5 injections of liposomal MDP-GDP (2 μg MDP-GDP in 1 μmol phospholipid) starting at day +3 and thereafter at 2–3 day intervals. For the prophylactic/therapeutic regimen, mice were injected via a lateral tail vein with 5×10^5 tumor cells. Treatment was started on day −3 prior to the tumor cell injection, continued at +4 or +24 h and thereafter at 2–3 day intervals for a total of 5 injections. The animals were sacrificed on day +20–+21 for both treatment protocols, and the number of liver tumors determined.

Statistical analysis. The effect of treatment on the number of hepatic tumors was determined using a computer-based Mann-Whitney U-Test [23]. The effect of treatment on the tumoricidal activity of Kupffer cells was determined using Student's *t*-test for unpaired data [23].

Results

Tissue distribution of DSPC/DMPG liposomes

Liposomes composed of DSPC/DMPG were efficiently localized within the liver after i.v. administration. Only a small proportion were localized within the lungs, and these were lost by 24 h after treatment with a concomitant increase in the amount localized within the liver (Table 1).

Incorporation of the lipophilic fluorescent probe NBD-PE within the liposome preparations resulted in a uniform 100% localization within the Kupffer cells. No fluorescence was observed in the parenchymal cell fraction or in the endothelial cells within the nonparenchymal fraction.

Activation of Kupffer cell tumoricidal activity in situ

The tissue distribution study demonstrated that a substantial proportion of DSPC/DMPG liposomes were localized within the liver. The ability of these liposomes to activate Kupffer cell tumoricidal activity was therefore determined. Kupffer cells consistently showed 15%–25% spontaneous tumoricidal activity in the absence of exogenous stimulation. The results from the in situ treatment (Table 2) showed that liposomes containing 2 μg MDP-GDP were able to activate Kupffer cell tumoricidal activity against H-59 tumor cell targets. Control liposomes were ineffective in inducing such activity. The MDP at doses of 100 μg was unable to induce Kupffer cell tumoricidal activity.

Table 1. Tissue distribution of ^{14}C-DSPC-labeled liposomes[a]

Time of sacrifice	% Retention of phospholipid[b]				
	Liver	Lungs	Kidneys	Spleen	Blood
4 h	52±7[c]	13±2	2±2	2±1	4±2
24 h	59±3	1±1	2±1	3±2	<1

[a] Groups of 5 mice were injected with 1 μmol distearoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DSPC/DMPG) containing 2 μg *N*-acetylmuramyl-L-alanyl-D-isoglutamine glycerol dipalmitate (MDP-GDP) and 0.5 μCi ^{14}C-DSPC

[b] The results are expressed as the percentage of injected radiolabel/organ. The results for blood are expressed as the percentage of radiolabel/ml whole blood

[c] Mean ± SD

Table 2. In situ activation of Kupffer cell tumoricidal activity[a]

In situ treatment	Residual cpm ± SD	% Cytotoxicity	P[b]
H-59 tumor cells alone	2802±331	–	–
Control Kupffer cells	2276±288	18.8	–
MDP, 100 μg/mouse	2169±129	22.6	NS
Control liposomes	2148±193	23.3	NS
Liposomal MDP-GDP, 2 μg/mouse	1270±125	54.7	<0.001

[a] Groups of 3 mice were treated with phosphate-buffered saline (PBS) (control), MDP, control liposomes (1 μmol), or liposomal MDP at the doses indicated. Kupffer cell-mediated tumoricidal activity was determined as described in *Materials and methods*

[b] The difference in cytotoxicity between control-treated Kupffer cells and treated Kupffer cells was determined using Student's *t*-test for unpaired data

Therapeutic treatment of H-59 hepatic tumors

The effect of treating experimental hepatic metastases of H-59 tumor cell line with doses of liposomal MDP-GDP able to induce Kupffer cell tumoricidal activity is shown in Table 3. The inhibitory activity of liposomal MDP-GDP was related to the initial tumor cell inoculum and the resultant number of hepatic metastases. Thus when a low dose of tumor cells ($2-3 \times 10^5$) was injected, resulting in 1–31 tumors in the control group, a significant reduction in the number of tumors was observed in the treatment groups. Control liposomes did not affect the number of hepatic tumors. Liposomal MDP-GDP treatment had no therapeutic activity on the incidence or number of experi-

Table 3. Liposomal MDP-GDP treatment of H-59 liver tumors[a]

Treatment	Incidence of liver tumors	No. of tumors/liver Median (range)[b]
Experiment 1: 2×10^5 tumor cells injected		
PBS	5/5	16 (12–23)
Liposomal MDP-GDP	6/6	6 (2–10)[c]
Experiment 2: 3×10^5 tumor cells injected		
PBS	5/5	23 (17–31)
Control liposomes	6/6	21 (8–30)
Liposomal MDP-GDP	4/6	2 (2–8)[c]
Experiment 3: 5×10^5 tumor cells injected		
PBS	5/5	26 (13–45)
Liposomal MDP-GDP	4/4	51 (9–58)

[a] C57BL/6 mice were injected with the indicated number of tumor cells on day 0. Liposomal treatment was initiated on day +3. A total of 5 injections was given over a period of 2 weeks

[b] The number of liver tumors was determined on day +21

[c] Significantly different from PBS treatment (Mann-Withney U-test, $P < 0.05$)

Table 4. Prophylactic/therapeutic treatment of H-59 liver tumors with liposomal MDP-GDP[a]

Treatment	Incidence of liver tumors	No. of tumors/liver Median (range)[b]
Experiment 1.		
PBS	10/10	54 (21 – 77)
Control liposomes	10/10	59 (19 – 86)
Liposomal MDP-GDP	10/10	6 (1 – 13)[c]
Experiment 2.		
PBS	8/ 8	40 (12 – 93)
Control liposomes	10/10	52 (10 – 103)
Liposomal MDP-GDP	6/ 9	4 (0 – 6)[c]

[a] C57BL/6 mice received the first treatment 3 days prior to tumor cell injection (5×10^5 tumor cells). The second treatment was given 4 or 24 h following tumor cell injection. Treatment was continued at 2–3 day intervals for a total of 5 injections

[b] The number of liver tumors was determined on day +20

[c] Significantly different from PBS treatment (Mann-Whitney U-test, $P < 0.05$)

mental metastases when higher tumor cell inocula (5×10^5 tumor cells), resulting in up to 45 liver tumors, were used. The results of one representative experiment of three performed is shown in Table 3.

Prophylactic/therapeutic treatment of H-59 hepatic tumors
The effect of prophylactic/therapeutic treatment on the incidence of experimental hepatic metastases of tumor H-59 is shown in Table 4. Liposomal MDP-GDP was effective in reducing the number of tumors whereas control liposomes were without effect. As can be seen, prophylactic treatment was more effective than therapeutic treatment in that significant reductions in the number of tumor nodules was observed, even when the higher tumor cell inocula (5×10^5 tumor cells) was used (Table 4).

Discussion

The limited success to date of conventional cancer therapy in the treatment of disseminated malignancies has prompted repeated attempts to develop immunotherapeutic regimens which aim to activate host defenses in situ. Such strategies are often confounded by inimical pharmacokinetics or hyporesponsiveness to immunomodulators. The ability of liposomes to deliver incorporated materials to cells of the reticuloendothelial system provides a methodology for targeting immunomodulators to macrophage effector cells. Attention in recent years has focused on MDP as an agent which is able to stimulate macrophage or monocyte tumoristatic or tumoricidal activity. However, the prolonged exposure time required for the induction of such activity by MDP [22] and its rapid excretion after parenteral administration [2, 13] render it ineffective in inducing such activity in vivo [10, 15, 17]. Liposomal encapsulation of MDP results in a significant enhancement of its ability to induce macrophage tumoricidal activity in vitro [6, 15, 22]. More importantly, such liposomes alter the pharmacokinetic behavior of MDP [11] and have been shown to inhibit the growth of mouse pulmonary metastases in vivo [10]. The introduction of lipophilic derivatives of MDP which are efficiently retained in liposomes and which possess antitumor activity against pulmonary tumors [9, 12, 15, 16, 17] represents a significant advance in immunopharmacology.

The results of this study demonstrate that DSPC/DMPG liposomes containing a lipophilic MDP, MDP-GDP, are capable of inducing Kupffer cell tumoricidal activity in situ after i.v. administration. The failure of free MDP at doses of 100 μg to induce such activity clearly illustrates the utility of liposomes as vehicles for targeting muramyl peptides to macrophage effector cells in the liver. The tissue distribution of DSPC/DMPG liposomes is such that considerable specificity of targeting to the liver is obtained.

Liposomal MDP-GDP was effective in reducing the number of hepatic tumors when administered in either a prophylactic/therapeutic or a therapeutic treatment protocol. Its failure to inhibit both the incidence and number of tumors in animals receiving a high tumor cell inoculum after therapeutic treatment suggests that liposomal immunotherapy, at least with MDPs, may only be effective when the liver tumor burden is below a certain limit. Such a limitation would not appear to apply for the prophylactic/therapeutic treatment of experimental metastases with liposomal MDP-GDP. The ability of these liposome preparations to deal with limited liver tumor burdens would suggest a clinical application in the treatment of those malignancies which have a propensity to metastasize to the liver (colorectal cancer, ocular melanoma) prior to the detection of such tumors.

All mice challenged with H-59 tumor cell line developed pulmonary tumors. The tumor load in the lungs (150– > > 250 tumors) was directly proportional to the challenge dose, and was unaffected by treatment with liposomal MDP-GDP. The low level of liposomal localization in the lungs observed with liposomes formulated from DSPC/DMPG in the molar ratio of 10:1 was insufficient to induce alveolar macrophage tumoricidal activity (N. C. Phillips, unpublished data): the activation of Kupffer cells by MDP-GDP incorporated within such liposomes provides further evidence that tissue-fixed macrophages are responsible for the observed antitumor activity.

Recent studies have shown that the ability of liposomal MDP-GDP to activate Kupffer cell tumoricidal activity in situ was strongly dependent on the dose and time of treatment, optimal activity (magnitude and duration of tumoricidal activity) being found with 1–2 μg MDP-GDP [18]. In contrast, MDP, while able to activate Kupffer cell tumoricidal activity in vitro in the dose range 20–200 μg/ml, was unable to induce Kupffer cell tumoricidal activity in situ at doses of up to 1000 μg. The results of the present study clearly demonstrate the enhanced activity of liposomal MDP-GDP compared with free MDP in inducing such activity.

The observation that the reticuloendothelial system can be functionally paralyzed by the repeated administration of high doses of liposomes [1] illustrates that their potential clinical use as carriers for immunomodulators affecting Kupffer cells will require careful consideration of dose and timing.

We are presently using various doses and schedules of inoculation to determine the therapeutic potency of liposomal MDP-GDP in the treatment of spontaneous hepatic metastases following the local removal of primary s.c. H-59 tumors.

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