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Increased Local Antitumor Effects of Interleukin 2 Liposomes in Mice with MCA-106 Sarcoma Pulmonary Metastases[1]

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ABSTRACT

The effects of liposome formulations of interleukin 2 (IL-2) and local route were studied in C57BL/6 mice with MCA-106 sarcoma pulmonary metastases. IL-2 liposomes made by hydration of powdered dimyristoylphosphatidylcholine with aqueous recombinant IL-2 had 95% of the IL-2 associated with the lipid fraction. When mice with pulmonary micrometastases were treated once daily with free cytokine on days 5, 6, and 7 after tumor inoculation, the intrathoracic route was superior to the i.p. or s.c. routes. When IL-2 liposomes were administered by the local intrathoracic route, significantly better antitumor effects ($P < 0.01$) were seen compared to empty liposomes or free IL-2 as determined by (*a*) increased survival and (*b*) reduced numbers of pulmonary metastases. Minimal toxicity was observed. Results indicate that local route and incorporation of IL-2 in liposomes may enhance therapeutic efficacy and facilitate more practical daily dosing regimens.

INTRODUCTION

IL-2,[2] a M_r 15,000 hydrophobic protein produced by helper T-lymphocytes, occupies a central role in the augmentation of cell-mediated immune responses (1). IL-2 also facilitates nonspecific tumor killing by activated macrophages (2) and induction of the lymphokine-activated killer phenomenon in lymphocytes (3–7). IL-2, alone or in combination with adoptively transferred cells, has effectively reduced the number of metastases in murine tumor models (8–10) and has been utilized with limited success in human cancer immunotherapy protocols (11–16). Major problems associated with high dose, prolonged IL-2 administration in humans have been related to severe systemic toxicity. Side effects have included fever, malaise, hepatic and renal dysfunction, and generalized edema associated with a life-threatening pulmonary capillary leak syndrome (15, 16).

Liposomes, lipid vesicles with one or many bilayers, can profoundly modify toxicity by altering the absorption and distribution of entrapped drugs by virtue of lymphatic absorption and macrophage uptake which occurs mainly in the liver and to a lesser extent in the lung, bone marrow, and spleen (17). For example, it has been elegantly shown (18) that the antifungal efficacy and systemic toxicity of amphotericin B are significantly improved by using a liposomal formulation. Therefore, liposomes containing IL-2 were synthesized and administered either systemically or locally to test their efficacy against pulmonary metastases in mice.

MATERIALS AND METHODS

IL-2 Liposome Synthesis. IL-2 (Hoffmann LaRoche; specific activity, 1.5×10^7 units/mg) containing 25 mg human serum albumin/10^6 units IL-2 or free of carrier protein was diluted in 0.9% NaCl solution or Hanks' balanced salt solution and the aqueous solution was added to DMPC (Avanti Polar Lipids, Pelham, AL) and mixed on a vortex apparatus. Freeze/thawing was done using three 5-min cycles in a dry ice/ethanol bath to freeze and a 37°C water bath to thaw. Brief bath sonication after each thaw cycle was utilized to improve IL-2 incorporation. The percentage of IL-2 in the liposome fraction was determined by CTLL-20 bioassay (19) and/or fluorescamine protein assay (20) of the liposome pellet and supernatant of preparations centrifuged at 1000 $\times$ *g* for 10 min. Endotoxin content was not detectable at 1:20 dilution in the *Limulus* lysate assay (E-Toxate; Sigma).

Liposomes were also made by detergent dialysis according to the method of Zumbuehl and Weder (21) using the Liposomat apparatus (Dianorm, West Germany).

Murine Pulmonary Metastatic Model. Groups of 10 C57BL/6 mice were housed and fed *ad libitum* according to University of Minnesota Research Animal Resources guidelines. Pulmonary metastases were induced by i.v. tail vein injection of 5×10^5 MCA-106 sarcoma cells in 0.4 ml Hanks' balanced salt solution. MCA-106 sarcoma, methylcholanthrene-induced tumor with slight immunogenicity, was developed by Dr. J. J. Mulé and Dr. S. A. Rosenberg at the National Cancer Institute and was obtained from Dr. R. Simmons. On days 5, 6, and 7 after tumor inoculation, mice received ether anesthesia and therapeutic injections of 0.2 ml free or liposomal IL-2 s.c., i.p., i.v., or i.tx. The number of pulmonary metastases was enumerated on day 14 or 15 after sacrificing mice in a CO_2 gas chamber, tracheal instillation of India ink, and storage of harvested lungs in Fekete's solution (300 ml 70% ethanol, 30 ml formaldehyde, and 15 ml glacial acetic acid) as described previously (22). Treatment results were compared to the control group using Student's unpaired *t* test.

RESULTS

IL-2 liposomes were first made using the same method and combination of lipids as in studies of amphotericin B liposomes in humans (18). Thus, MLV containing DMPC and DMPG in a 7:3 ratio were made by standard aqueous hydration. Using a lipid:protein ratio of 300:1, IL-2 MLV had low and variable entrapment of IL-2 (10–50%). The detergent dialysis technique (21) resulted in unilamellar vesicles with only 42% of IL-2 entrapped. Moreover, this method was cumbersome taking several days and requiring a specialized apparatus. Freeze-thawing of DMPC/DMPG MLVs containing IL-2, however, reliably resulted in higher IL-2 protein entrapment as measured by either the IL-2 bioassay or the fluorescamine protein assay. This is consistent with the observation that the freeze-thaw procedure increases the aqueous space available in multilamellar vesicles 5–10-fold (23).

Superior incorporation of IL-2 in liposomes was obtained with DMPC alone. Furthermore, when the hydration and freeze-thaw procedure was used with bath sonication, over 95%percent of IL-2 was incorporated. The effects of IL-2 protein and lipid concentrations, freezing and thawing cycles, and brief (30 s) bath sonication are detailed in Table 1. Liposome IL-2 preparations had no detectable leakage of contents when stored at 4°C for 3 months. If made in the presence of 125 mM sucrose or trehalose, diluted IL-2 liposome prepara-

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[2] The abbreviations used are: IL-2, interleukin 2; DMPC, dimyristoylphosphatidylcholine; i.tx., intrathoracic; MLV, multilamellar vesicles; DMPG, dimyristoylphosphatidylglycerol; IL-6, interleukin 6.

Table 1 *Method of IL-2 liposome synthesis*

Important variables included lipid and protein concentration, freeze-thawing, and bath sonication.

With carrier protein (HSA)	% IL-2 incorporation	
	50 mg DMPC/ml[a]	300 mg DMPC/ml[b]
Method of liposome synthesis		
1. Aqueous hydration (vortex mixer)	31	70
2. Hydration, then freeze-thaw × 3	43	91
3. Hydration, freeze-thaw, and bath sonicate × 3	54	97
Without carrier protein (hydration, freeze-thaw, and bath sonicate × 3)	**Lipid/IL-2 ratio**	**% IL-2 incorporation**
	30:1	17.2
	100:1	37.5
	150:1	86.2
	200:1	94.6

[a] With HSA carrier protein, lipid/protein ratio = 2:1 in 50 mg DMPC/ml; lipid/IL-2 ratio = 750:1 in 50 mg DMPC/ml.
[b] With HSA carrier protein, lipid/protein ratio = 12:1 in 300 mg DMPC/ml; lipid/IL-2 ratio = 4500:1 in 300 mg DMPC/ml.

tions could be frozen without loss of vesicle contents when thawed.

Bioavailability of IL-2 in liposomes was similar to the free cytokine in the IL-2 bioassay which measures [^{3}H]thymidine incorporation into DNA of the IL-2-dependent CTLL-20 cell line (19). The serum clearance time (*i.e.*, elimination $t_{1/2}$) of s.c. IL-2 liposomes in C57BL/6 mice was 68 min compared to 6 min for the free drug. The marked depot effect of this formulation was demonstrated by the detection of 10 units of IL-2 in the serum of mice 72 h after a single s.c.,) injection of 250,000 units of IL-2 liposomes whereas free drug could not be detected at 24 h. These results are similar to depot effects of liposomes with hemagglutinin protein antigens (24). The elimination $t_{1/2}$ of i.p. free IL-2 was determined to be 24 min with only trace amounts being present 6 h following injection of 50,000 units of IL-2; in contrast, the elimination $t_{1/2}$ of IL-2 liposomes after i.p. injection was 4.3 h and IL-2 was detectable in the bioassay at 24 h.

Cure of mice with s.c. MCA-106 sarcoma was seen after local injection of IL-2 liposomes but not free IL-2 directly into the slightly immunogenic MCA-106 tumor (data not shown). Previous studies (5) and work by us (22) have shown significant tumor reduction with repeated three times/day i.p. injections of free IL-2. However, when the antitumor activity was tested using the i.p. route and once daily schedules, neither free IL-2 nor IL-2 liposomes had significant therapeutic effects.

In contrast, markedly increased efficacy of local IL-2 was demonstrated against pulmonary metastases using the local i.tx. route (Tables 2–4; Fig. 1). Injection i.tx. of either free IL-2 or IL-2 liposomes into the right side of the chest resulted in reduction of lung metastases in both lungs. In 3 of 10 comparisons of i.tx. free *IL-2 versus* i.tx. IL-2 liposomes no significant difference was seen between treatment groups (Table 2; Table 4, Experiment 2, $P = 0.203$, 0.058, and 0.118, respectively). Notably none of 10 comparisons was the free IL-2 treatment significantly better than IL-2 liposomes.

Using the local i.tx. route and a daily for 5 days schedule of i.tx. IL-2 injections, a dose response of IL-2 liposomes was demonstrated; IL-2 liposome formulation reduced pulmonary metastases significantly better than free cytokine at all doses tested in this experiment (Table 3; $P = 0.002$). In another experiment (Table 4, Experiment 1) an escalating dose schedule resulted in significantly fewer metastases in the group treated with i.tx. IL-2 liposomes than i.tx. free cytokine ($P = 0.020$).

Table 2 *Effect of local route of IL-2 in the murine MCA-106 sarcoma pulmonary metastasic model*

Pulmonary metastases were induced in groups of 10 C57BL/6 mice by i.v. tail vein injection of 5×10^5 MCA-106 sarcoma cells in 0.4 ml Hanks' balanced salt solution. On days 5, 6, and 7 (Experiment A) or days 5–9 (Experiment B) after tumor inoculation, mice received anesthesia and therapeutic injections of 50,000 units free or liposomal IL-2 by the following routes: s.c., i.p., i.v., or directly into the right chest, *i.e.*, i.tx. Treatment results were compared to the no therapy control group (Experiment A).

Route/treatment	No. of pulmonary metastases			
	Mean	Median	SD	*P*
Experiment A				
None	137	137	39	
i.v. IL-2 liposomes	99	91	54	0.097
s.c. free IL-2	77	86	29	0.001
s.c. IL-2 liposomes	79	70	45	0.013
i.tx. free IL-2	23	10	30	0.001
i.tx. IL-2 liposomes	11	6	10	<0.001
Experiment B				
i.tx. saline	193	197	71	
i.tx. empty liposomes	144	146	66	0.094[a]
i.p. free IL-2	123	142	69	0.416[b]
i.p. IL-2 liposomes	153	164	81	0.762[b]
i.v. free IL-2	129	120	67	0.563[b]
i.v. IL-2 liposomes	153	157	73	0.759[b]
i.tx. free IL-2	68	72	27	0.004[b]
i.tx. IL-2 liposomes	32	8	46	<0.001[b]

[a] i.tx. saline using Student's unpaired *t* test.
[b] i.tx. empty liposome groups (Experiment B) using Student's unpaired *t* test.

Table 3 *Comparison of antitumor effects of IL-2 liposomes and free cytokine on murine pulmonary metastases*

Groups of 10 C57BL/6 tumor-bearing mice were treated daily for 5 consecutive days with IL-2 using i.tx. free IL-2 or i.tx. liposome IL-2 formulations on days 4–8 after i.v. MCA-106 sarcoma tumor inoculation. IL-2 formulations were given by the local intrathroacic route in 0.2 ml. Comparisons of treatment results between free and liposome IL-2 formulations were done using Student's *t* test.

IL-2 dose (units)	No. of MCA-106 sarcoma lung metastases				
	IL-2 liposomes		free IL-2		
	Mean	SD	Mean	SD	*P*
None			91.7	21.3	
10,000	34.5	17.9	75.9	30.9	0.002
25,000	19.9	13.1	59.8	20.7	0.001
50,000	9.6	4.5	65.3	40.0	0.002

Table 4 *Antitumor effect of 3 consecutive daily doses of intrathoracic IL-2 liposomes on pulmonary metastases*

Groups of 8–10 C57BL/6 tumor-bearing mice were treated daily with local IL-2 using i.tx. free IL-2 or i.tx. liposome IL-2 formulations on days 5, 6, and 7 after MCA-106 sarcoma tumor inoculation. Treatment results evaluated by direct counting of day 15 lung metastases. Results were compared using Student's *t* test compared to control (no therapy) groups.

Treatment	IL-2 dose once daily qd for 3 days	No. of metastases			
		Mean	Median	SD	*P*
Experiment 1					
No therapy		231.9	250	51	
Free IL-2	150,000 units, day 5 100,000 units, day 6 50,000 units, day 7	107.7	68.5	94	0.008
IL-2 liposomes	150,000 units, day 5 100,000 units, day 6 50,000 units, day 7	21.3	7	26	<0.001
Experiment 2					
No therapy		236	224	40	
Free IL-2	100,000 units	20	5	30	<0.001
IL-2 liposomes	100,000 units	3.1	1	5.1	<0.001

In 3 of 3 groups of mice treated with IL-2 liposomes significantly improved survival was seen compared to mice receiving the free cytokine (life table analysis log rank test statistic = 0.016, 0.042, and 0.047; Fig. 1, *top, middle,* and *bottom,* respectively). In summary, in 7 of 10 direct comparisons of i.tx. free IL-2 and i.tx. IL-2 liposomes, the latter had significantly increased antitumor effects against pulmonary metastases.

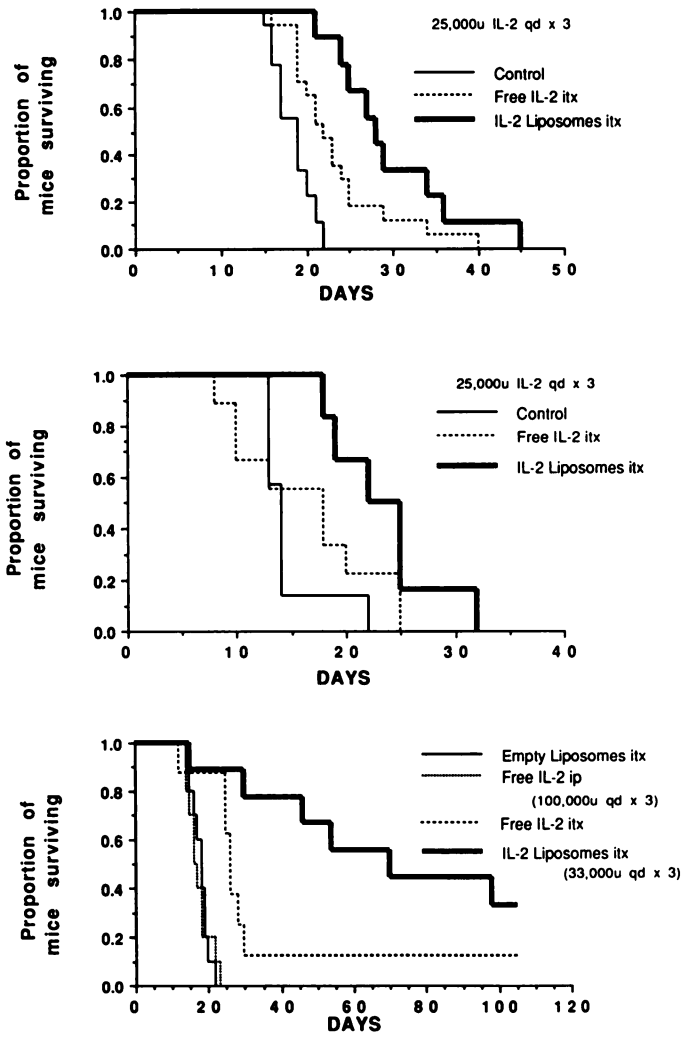

Fig. 1. Immunotherapy of MCA-106 pulmonary metastases with daily IL-2. Effects of route and liposome formulation on survival. Mice received daily injections of IL-2 at doses indicated on days 5, 6, and 7 after tumor inoculation as described in Table 2. Control groups received no therapy (*top, middle*) or empty Hanks' balanced salt solution-loaded liposomes at the same lipid dose, 3 mg DMPC/injection, as the IL-2 liposome group (*bottom*). Survival of mice receiving i.tx. IL-2 liposomes was significantly improved compared to control (log rank test $P < 0.001$, 0.009, and < 0.001, *top, middle,* and *bottom,* respectively). IL-2 liposome groups also had significantly increased survival compared to free IL-2 groups (log rank test $P < 0016$, 0.042, and 0.047).

DISCUSSION

IL-2 liposomes were made in about 30 min using stable saturated synthetic lipids which required no special storage other than desiccation at −20°C. Thus, IL-2 liposomes could be easily made with this procedure by most laboratories or hospitals. The phase transition temperature (*Tm*) of the lipids used in this study, DMPC and DMPG, is 23°C; the membranes of these IL-2 liposomes presumably are "solid" when stored at 4°C but "fluid" *in vivo*. This as well as the size (M_r 15,000) and amphipathic nature of IL-2 may contribute to the low leakage of IL-2 which was observed at 4°C. Although rapid macrophage uptake and degradation of liposomes may interfere with the antitumor activity of systemic IL-2 liposome routes (*e.g.,* i.v. or i.p. for pulmonary metastases), the ability of locally administered IL-2 liposomes to mediate antitumor activity was documented in numerous separate murine experiments.

At the present time the mechanism(s) of action of the local antitumor effects of IL-2 liposomes or free IL-2 are not precisely known. In previous studies, repeated 3 times daily injections of IL-2 have been effective in the reduction of pulmonary metastases of MCA-106 sarcoma (6, 22). In this study once daily i.p. bolus regimens had no significant antitumor effects, probably related to rapid clearance of the drug. Using once daily schedules, IL-2 in liposomes had superior antitumor effects compared to the free cytokine. Possible mechanisms of increased activity of IL-2 liposomes could include slow release of IL-2 and/or longer half-life, activation of macrophages to become tumoricidal, lymphatic uptake of liposomes (17, 25–28), more efficient stimulation of specific cytotoxicity or the lymphokine-activated killer phenomenon in lymphocytes, or production of secondary cytokines such as IL-6 or tumor necrosis factor. IL-6 is an acute phase reactant which has synergistic effects with IL-2 in the activation of specific cell-mediated immune responses (29, 30) and has been shown to be increased following IL-2 administration (31).

Since the half-life of IL-2 in the peritoneal cavity is prolonged compared to the free formulation, the same is probably true of pleural (i.tx.) administration of IL-2 in liposomes. From this site the drug is probably absorbed via the pulmonary lymphatics. The amount of bioactive IL-2 available in the liver or pulmonary parenchyma (including hematogenously disseminated metastases) could not be reliably determined due to the inhibition of growth of indicator CTLL-2 or CTLL-20 cell lines by tissue homogenates. Commercially available IL-2 enzyme-linked immunoassay kits were not sensitive for these types of studies. Nevertheless, it may be possible to increase the sensitivity of IL-2 enzyme immunoassay using procedures such as those described by Budd and Smith (32) to permit tissue pharmacokinetic analysis.

In the murine studies using once daily i.p. or i.tx. dosing regimens as described in this report, no obvious signs of severe systemic toxicity of IL-2 were seen as determined by ruffled coat or decreased activity level. Further analysis of IL-2 liposome toxicity will be complex because the toxicity of IL-2 liposomes is dose, route, and schedule dependent. Quantitation of pulmonary capillary leak, the dose-limiting toxicity of IL-2, has been done by others (33, 34) and will be the subject of future studies in our laboratory.

Advantages of local route and IL-2 liposome formulation in a murine tumor model were detailed in this report. Since local intrapleural free IL-2 has been reported to reduce malignant pleural effusions in lung cancer patients (35), this route is not without precedent. Although the antitumor efficacy of our approach, *i.e.,* IL-2 liposomes administered locally once daily, remains to be determined in humans, such a regimen has potential for the development of human outpatient treatment protocols.

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