Influence of Vesicle Size, Lipid Composition, and Drug-to-Lipid Ratio on the Biological Activity of Liposomal Doxorubicin in Mice

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Influence of Vesicle Size, Lipid Composition, and Drug-to-Lipid Ratio on the Biological Activity of Liposomal Doxorubicin in Mice[1]

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ABSTRACT

The effects of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice have been investigated using a versatile procedure for encapsulating doxorubicin inside liposomes. In this procedure, vesicles exhibiting transmembrane pH gradients (acidic inside) were employed to achieve drug trapping efficiencies in excess of 98%. Drug-to-lipid ratios as high as 0.3:1 (wt:wt) could be obtained in a manner that is relatively independent of lipid composition and vesicle size. Egg phosphatidylcholine (EPC)/cholesterol (55:45; mol/mol) vesicles sized through filters with a 200-nm pore size and loaded employing transmembrane pH gradients to achieve a doxorubicin-to-lipid ratio of 0.3:1 (wt/wt) increased the LD_{50} of free drug by approximately twofold. Removing cholesterol or decreasing the drug-to-lipid ratio in EPC/cholesterol preparations led to significant decreases in the LD_{50} of liposomal doxorubicin whereas, the LD_{50} increased 4- to 6-fold when distearoylphosphatidylcholine was substituted for EPC. The results suggest that the stability of liposomally entrapped doxorubicin in the circulation is an important factor in the toxicity of this drug in liposomal form. In contrast, the antitumor activity of liposomal doxorubicin is not influenced dramatically by alterations in lipid composition. Liposomal doxorubicin preparations of EPC, EPC/cholesterol (55:45; mol:mol), EPC/egg phosphatidylglycerol (EPG)/cholesterol (27.5:27.5:45; mol:mol), and distearoylphosphatidylcholine/cholesterol (55:45; mol:mol) all demonstrated similar efficacy to that of free drug when given at doses of 20 mg/kg and below. Higher dose levels of the less toxic formulations could be administered, leading to enhanced increase in life span (ILS) values. Variations in vesicle size, however, strongly influenced the antitumor activity of liposomal doxorubicin. At a dose of 20 mg/kg, large EPC/cholesterol systems are significantly less effective than free drug (with ILS values of 65% and 145%, respectively). In contrast, small systems sized through filters with a 100-nm pore size are more effective than free drug, resulting in an ILS of 375% and a 30% long term (greater than 60 days) survival rate when administered at a dose of 20 mg/kg. Similar size-dependent effects are observed for distearoylphosphatidylcholine/cholesterol systems.

INTRODUCTION

Doxorubicin is a potent antineoplastic agent active against a wide range of human neoplasms. However, administration of this drug is associated with severe acute toxicities (including myelosuppression and gastrointestinal toxicity) as well as a cumulative dose-limiting cardiotoxicity (1). Many reports in various animal models reveal a consistent ability of liposome encapsulation to ameliorate both acute and chronic toxic side effects of doxorubicin. For instance, the cumulative and dose-limiting cardiotoxicity associated with use of free doxorubicin can be reduced by presenting the drug in encapsulated form (2–8). In addition, other indicators of toxicity, such as weight loss (6, 8), alopecia (6) and urinary albumin concentration (8, 9), as well as dermal necrosis resulting from extravasation (10) can be reduced or eliminated by employing liposomal doxorubicin. This reduction in toxicity has been correlated with decreased drug accumulation in tissues associated with the respective toxicities (2, 4, 5, 7, 10). Various ascitic (4, 5, 7, 11), metastatic (12–15), and solid tumor (9, 16–18) model studies have demonstrated that this buffering of toxicity is not obtained at the expense of antitumor efficacy.

While these investigations establish the clinical potential of liposome-encapsulated doxorubicin, clear indications of the optimal liposome preparation to employ and the mechanism by which liposomes alter the therapeutic activity of doxorubicin are not so readily available. The difficulties associated with identifying an optimal system are implicit in the wide range of vesicle types, lipid compositions, and drug-to-lipid ratios previously employed (see Table 1). In particular, MLVs,[4] LUVs, and SUVs have been utilized with lipid compositions incorporating varying amounts of positively charged and negatively charged lipids in addition to PC and cholesterol. The resulting drug-to-lipid ratios vary by a factor of 17 and trapping efficiencies range from 4 to 90% (Table 1). The variations in lipid composition largely stem from the requirements for trapping doxorubicin, as systems containing only positively charged or neutral lipids exhibit low trapping efficiencies and drug-to-lipid ratios (Table 1). In liposomes containing negatively charged lipids, such as cardiolipin and phosphatidylglycerol, higher drug-to-lipid ratios can be achieved because of the association of the positively charged doxorubicin with the negatively charged lipids (Table 1). However, inclusion of negatively charged lipids changes the vesicle surface charge and may affect biodistribution and clearance properties (19). Furthermore, alterations in lipid composition can affect the size distributions achievable for certain liposomal doxorubicin systems (17). Consequently, a comprehensive profile of the effects of liposome characteristics on the therapeutic activity of liposomal doxorubicin has not been achieved.

We have previously described the use of transmembrane pH gradients to efficiently encapsulate high levels of doxorubicin inside liposomes (20). This encapsulation procedure does not require the presence of negatively charged lipids and is highly flexible. Initial studies employing EPC/cholesterol-encapsulated doxorubicin demonstrated that such preparations exhibit the reduced toxicity associated with previous liposomal systems (21). Here we use such systems to investigate the influence of liposome size, lipid composition, and drug-to-lipid ratio on

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[4] The abbreviations used are: MLV, multilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; PC, phosphatidylcholine; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearylphosphatidylcholine; ILS, increase in life span; QELS, quasielastic light scattering; HPLC, high pressure liquid chromatography; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; GOT, glutamate oxaloacetic aminotransferase; AP, alkaline phosphatase.

Table 1 *Characteristics of liposome encapsulated-doxorubicin preparations*

Ref.	Liposome type	Size (nm)	Composition	Ratio	Drug:lipid mol:mol	Drug:lipid wt:wt	Trapping efficiency (%)
12	SUV	135 ± 70	PS:PC:C[a]	3:7:10	1:18.6	0.05:1	25
4	MLV	ND[b]	PC:C	1:1	1:33	0.028:1	14
	MLV	ND	PC		1:31.2	0.022:1	10
	MLV	ND	CL:PC:C	1:4:5	1:21.2	0.039:1	62
	MLV	ND	CL:PC	1:4	1:14.8	0.040:1	58
	MLV	ND	PS:PC:C	3:7:10	1:23	0.040:1	42
	SUV	ND	PC:C	1:1	1:14	0.066:1	15
	SUV	ND	CL:C	5:2:5	1:18	0.027:1	90
	SUV	ND	CL:PC:C	1:4:2	1:21.2	0.033:1	47
	SUV	ND	CL:PC:C	1:4:5	1:26.7	0.031:1	45
	SUV	ND	PS:PC:C	3:7:10	1:44.2	0.021:1	22
18	SUV	ND	PC:C	7:2	1:130	0.006:1	7
	SUV	ND	PC:C:DCP	7:2:1	1:37	0.021:1	26
	SUV	ND	PC:C:SA	7:2:1	1:225	0.004:1	4
2	SUV	ND	PC:C:PS	10:4:1	1:11.6	0.069:1	55
	SUV	ND	PC:C:SA	10:4:3	1:18.4	0.049:1	35
6	SUV	90 ± 20	CL:PC:C:SA	1:5:3.5:2	1:12.4	0.068:1	55
5	LUV	150	PG:PC:C	1:4:5	1:30	0.031:1	50
8	SUV	75 ± 27	PC:PG:C	7:3:4	1:14	0.055:1	50
17	LUV	300	PC:PS:C	10:1:4	1:20.4	0.039:1	57
	LUV	730	DPPC:DPPG:C	10:1:10	1:43.2	0.022:1	27

[a] PC, phosphatidylcholine; PS, phosphatidylserine; C, cholesterol; CL, cardiolipin; DCP, dicetylphosphate; SA, stearylamine; PG, phosphatidylglycerol.
[b] ND, not determined.

doxorubicin toxicity and antitumor efficacy in a systematic manner.

MATERIALS AND METHODS

Materials

EPC, EPG, and DSPC were purchased from Avanti Polar Lipids and were greater than 99% pure. Doxorubicin was purchased from Adria Laboratories. Cholesterol, citric acid, Na_2CO_3, and NaOH were of reagent or USP grade. All mice were purchased from Charles River Breeding Laboratories.

Methods

Liposomal Preparation. Vesicles were prepared by hydrating a lipid film (dried down from $CHCl_3$ for 12 h under high vacuum) in the presence of 300 mM citric acid (pH 4.0) to achieve lipid concentrations of 100 mg to 200 mg total lipid/ml. The MLVs were then frozen and thawed five times as described previously (22) and extruded 10 times through polycarbonate filters of the indicated pore size employing a liposome extruder obtained from Lipex Biomembranes (Vancouver, BC). For DSPC/cholesterol vesicles, extrusion was conducted at 65°C. Vesicle size distributions were determined by QELS employing a Nicomp model 270 particle sizer. Vesicle morphology and size were monitored by freeze-fracture electron microscopy employing a Balzers BAF 400D freeze-fracture apparatus and a Phillips 400 electron microscope as described previously (23, 24).

Doxorubicin Encapsulation. Extruded vesicles were diluted twofold where needed with sterile saline to achieve a total lipid concentration of 100 mg/ml. This step was necessary to avoid death on injection caused by high citric acid concentrations at i.v. doses of liposomal doxorubicin in excess of 70 mg doxorubicin/kg. The exterior pH of these vesicles was then titrated to 7.8 with 1.0 M NaOH or 1.0 M Na_2CO_3, thus creating a pH gradient (acidic inside) across the vesicles. This vesicle solution and powdered doxorubicin were then heated at 60°C for 3 min, combined at the indicated drug-to-lipid ratio and heated at 60°C for 10 min with intermittent vortex mixing. Vesicle-entrapped doxorubicin was determined by column chromatography methods as described previously (20). Thin-layer chromatography and HPLC analysis indicated that no degradation of doxorubicin occurred under the encapsulation conditions.

In vitro stability of liposome encapsulated doxorubicin was tested by dialyzing samples (2 mM to 10 mM lipid) for 24 h against 1000 volumes of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl (pH 7.5) at 37°C. At the indicated times, 150-μl aliquots were removed and entrapped doxorubicin was determined by column chromatography as described above.

Survival Studies. The toxicity of various doxorubicin formulations was investigated by determining the apparent LD_{50} of the drug administered in 0.2 ml via tail vein injection to female CD1 mice (20–25 g body weight). Groups of 10 mice per dose were monitored over 14 days, deaths were noted and mean weights were determined on Day 7 (which was generally the weight-loss nadir) for surviving mice. Individual experiments were repeated as appropriate to allow direct comparisons to be made. LD_{50} values and 95% confidence intervals were determined by logistic dose response analysis as described by Williams (25). Calculations were performed utilizing generalized linear modeling.

Tissue Distribution Studies. Doxorubicin, as free drug or in liposome-encapsulated form, was injected via a tail vein at a dose of 20 mg/kg body weight to groups of five female CD1 mice (20–25 g body weight). At the indicated times postinjection the mice were sacrificed by cervical dislocation, blood was collected by heart puncture, and the liver, heart, lungs, kidney, and spleen were immediately removed. For blood clearance kinetic studies, blood was removed from the carotid artery. Tissues were lightly blotted to remove any excess blood and weighed within 3 min of excision. These tissues, as well as 0.2 ml blood, were then lyophilized and analyzed for doxorubicin and its fluorescent metabolites according to the methods of Bachur *et al.* (26). Briefly, 3 ml 50% aqueous acidic ethanol (0.3 N HCl) was added to lyophilized or frozen samples and homogenized for 2 min using a Brinkmann Instrument Polytron. The resulting homogenates were incubated for 3 h at 4°C in the dark and then centrifuged at 15,000 × *g* for 30 min. The supernatants were analyzed for doxorubicin and its fluorescent metabolites by monitoring the fluorescence intensity at 590 nm employing an SLM-Amino SPF-500C spectrofluorometer and comparing with standard samples containing known amounts of doxorubicin that had been processed in the same manner. Control tissues were also monitored to correct for endogenous fluorescence. Corrections for tissue blood volumes were made on the basis of ^{51}Cr red blood cell distributions in the studied organs of CD1 mice.

Serum Enzyme Analysis. Serum enzyme determinations were conducted on Days 1 and 10 postinjection (five mice per timepoint for each group). Blood samples (approximately 1 ml) were collected from the carotid artery and placed on ice for 30 min. The samples were then allowed to incubate at room temperature for 30 min, at which time

they were centrifuged to pellet the clot material. Serum was then removed and analyzed for creatine kinase, lactate dehydrogenase, alkaline phosphatase, and aspartate oxaloacetic aminotransferase enzyme activities by the Department of Laboratory Medicine at the Acute Care Unit Hospital (Vancouver, BC) employing Roche diagnostic kits.

Animals and Tumor Models. Female DBA/2 mice weighing 18–20 g were obtained from Charles River Breeding Laboratories. The L1210 cell line was obtained from J. Levy, Department of Microbiology, UBC, and was maintained by serial passage of ascites fluid or as a frozen (liquid N_2) culture. Mice, in groups of six to ten, were inoculated via i.p. injections of 1.5×10^6 L1210 tumor cells suspended in 0.5 ml RPMI 1640. Treatment was initiated 1 day after injection of tumor cells and was given as a single i.v. dose via the lateral tail vein. The animals were treated with free or liposomal doxorubicin at specified doses based on mean body weight. Control groups were treated with either sterile saline or empty liposomes at a lipid dose equivalent to that given with the highest dose of liposomal doxorubicin. Mice were weighed on the day before tumor injection, and weights were recorded daily until the first death within a group. Survival time was recorded in days after tumor injection. Mean and median survival times and statistical significance of the results were determined employing a two-tailed Wilcoxon's ranking test (randomized two-group design). Experiments always included free drug groups and liposomal doxorubicin preparations were repeated as required for appropriate comparisons to be made. All data obtained for repeated experiments were pooled and utilized for statistical analysis.

RESULTS

Characterization of Liposomal Doxorubicin Systems Prepared by Transmembrane pH Gradient Encapsulation Techniques

We have previously demonstrated the use of transmembrane ion gradients (20, 27) to "actively" entrap doxorubicin inside liposomes. Fig. 1 demonstrates that the rate and efficiency of drug encapsulation in response to pH gradients is extremely temperature dependent for liposomal systems containing cholesterol. Incubation of doxorubicin in the presence of liposomes with an imposed transmembrane ΔpH of 3.5 (acidic inside) at 21°C results in only 30% trapping efficiency after 90 min. By increasing the incubation temperature to 37°C or above, trapping efficiencies approaching 100% can be achieved within 90 min and 2 min for incubation temperatures of 37°C and 60°C, respectively. Under these conditions, thin-layer chromatography and HPLC analyses indicated no detectable doxorubicin breakdown (data not shown). Therefore, in all subsequent encapsulation studies liposomes were incubated with doxorubicin at 60°C for 10 min to maximize drug trapping efficiency. Further, although previous studies have indicated that increases in the drug-to-lipid ratio for some liposomal systems can lead to structural alterations in the vesicles (5), QELS and freeze-fracture electron microscopy analyses indicated no significant changes in size distribution (Table 2), and freeze-fracture planes revealed the presence of closed vesicular systems (data not shown).

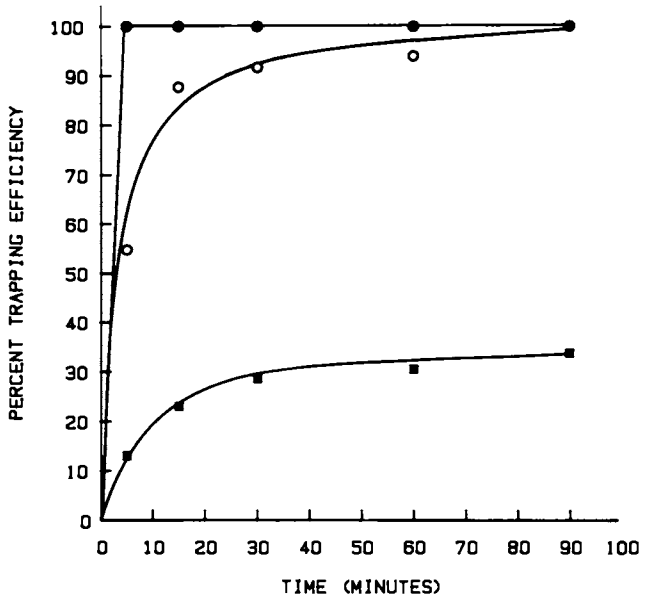

Fig. 1. Effect of incubation temperature on ΔpH-dependent doxorubicin uptake into EPC/cholesterol (55:45 mol ratio) vesicles. Vesicles were prepared in 300 mM citric acid (pH 4.0) and extruded through 200-nm pore size polycarbonate filters. Prior to doxorubicin addition, the external vesicle medium was brought to pH 7.8 with sodium hydroxide. Doxorubicin (3.0 mg/ml) was added to liposomes (11.0 mg lipid/ml) equilibrated at 21 (■), 37 (○), and 60°C (●). Entrapped doxorubicin was determined as described in "Materials and Methods."

The effects of vesicle size, lipid composition, and drug-to-lipid ratio on ΔpH-dependent doxorubicin uptake and retention were studied for various liposome systems. The results presented in Table 2 demonstrate that trapping efficiencies approaching 100% can be obtained for vesicles ranging in size from 0.10 to 1.4 μm, for drug-to-lipid ratios from 0.03:1 (wt:wt) to 0.3:1 (wt:wt), and for lipid compositions containing neutral, negatively charged or saturated phospholipids as well as varying amounts of cholesterol. Studies evaluating doxorubicin retention in vesicles (Table 2) indicate that typically less than 5% of the entrapped drug is released from PC/cholesterol (55:45; mol:mol) liposomes over 24 h at 37°C for a drug-to-lipid weight ratio of approximately 0.3:1. This finding contrasts with previous observations where between 20% and 50% of passively encapsulated doxorubicin is released from EPC/cholesterol liposomes within 1 h (4, 27). Decreasing the cholesterol content of EPC/cholesterol liposomes, as well as including the negatively charged phospholipid PG, results in increased drug leakage from liposomes after entrapment, consistent with earlier results (27).

Evaluation of Biological Activity

Role of Lipid Composition. The toxicities of free and liposomal doxorubicin preparations were ascertained by 14-day dose-response survival studies in CD1 mice. As shown in Table 3, an LD_{50} of 23 mg/kg is observed for free doxorubicin, which is comparable to that obtained in previous studies (7). This value is increased to 57 mg/kg when administered in liposome entrapped form (EPC/cholesterol, 55:45; 0.29 ± 0.02 mg doxorubicin/mg total lipid) in which the vesicles displayed a mean

Table 2 *Effect of liposome size, lipid composition, and drug-to-lipid ratio on ΔpH-dependent uptake and retention of doxorubicin in liposomes*

Vesicle-entrapped doxorubicin was determined as described in "Materials and Methods."

Lipid composition (molar ratio)	Vesicle size[a] mean ± SD (nm)	Drug/lipid (wt:wt)	Trapping efficiency %	% Retention after 24 h
EPC	158 ± 37	0.29:1	>99.0	53
EPC/C[b] (85:15)	166 ± 49	0.28:1	99.0	67
EPC/C (67:33)	163 ± 49	0.31:1	>99.0	86
EPC/C (55:45)	106 ± 31	0.25:1	98.8	>95
EPC/C (55:45)	160 ± 43	0.29:1	>99.0	>95
EPC/C (55:45)	1400 ± 400	0.28:1	>99.0	>95
EPC/C (55:45)	160 ± 48	0.038:1	>99.0	>95
DSPC/C (55:45)	773 ± 140	0.28:1	98.7	>95
DSPC/C (55:45)	175 ± 41	0.28:1	>98.9	>95
EPC/EPG/C (27.5:27.5:45)	180 ± 49	0.30:1	98.6	76

[a] Vesicle size was determined after doxorubicin entrapment employing quasielastic light scattering as described in "Materials and Methods."
[b] C, cholesterol.
[c] Retention of doxorubicin in liposomes was determined at 37°C as described in "Materials and Methods."

Table 3 *Effect of lipid composition on the toxicity of liposomal doxorubicin*

Vesicle-entrapped doxorubicin was prepared as described in "Materials and Methods." Doxorubicin-to-lipid ratios for all samples were 0.27 ± 0.04:1 (wt:wt).

Preparation	Mean vesicle diameter ± SD (nm)	LD_{50} (mg/kg)	95% Confidence interval (mg/kg)
Free		23	22–25
EPC/C[a] (55:45)[b]	160 ± 43	57	54–60
EPC/C (67:33)	163 ± 49	53	49–58
EPC/C (85:15)	166 ± 49	44	39–48
EPC	158 ± 37	38	33–42
DSPC/C (55:45)	175 ± 41	161	158–174
EPC/EPG/C (27.5:27.5:45)	180 ± 51	55	52–59

[a] C, cholesterol.
[b] Numbers in parentheses, molar ratios of lipid components.

diameter of 160 nm. Extension of the time course to 30 days and beyond had negligible effects on the relative toxicities of the free and liposomal drug forms. Doxorubicin toxicity is also revealed in the body weight change in mice 7 days after injection with doxorubicin (nadir in weight change for surviving mice). Dose-response curves similar to those for 14-day survival are obtained: a 20% weight loss is observed at 20 mg/kg and 60 mg/kg for free and EPC/cholesterol (55:45)-encapsulated doxorubicin, respectively (data not shown). Decreasing the cholesterol content of equivalently sized EPC systems from 45 to 0 mol % decreases the LD_{50} from 57 to 38 mg/kg (significantly different as indicated by the 95% confidence intervals). This dependence of doxorubicin toxicity on the cholesterol content of the vesicle is consistent with the cholesterol content required to avoid leakage of entrapped material into the circulation (28) and suggests that the extent of toxicity observed for liposomal doxorubicin is related to *in vivo* stability of the liposomes. This interpretation is supported by the observation that equivalently sized DSPC/cholesterol liposomal doxorubicin preparations exhibit LD_{50} values approximately threefold higher than EPC/cholesterol systems (Table 3). Incorporation of the negatively charged phospholipid phosphatidylglycerol into EPC/cholesterol liposomes does not appear to dramatically alter the toxicity of liposomal doxorubicin. Lethality studies for liposomal doxorubicin preparations administered to non-tumor bearing DBA/2 mice indicate that this mouse strain which is employed for efficacy evaluations as described below is more susceptible to liposomal doxorubicin preparations. Absolute LD_{50} values were lower in DBA/2 mice for EPC/cholesterol and DSPC/cholesterol systems (40 and 70 mg/kg, respectively; data not shown), while that of free doxorubicin remained essentially unchanged. However, the relative toxicities observed between the two liposomal formulations are consistent with the results obtained in CD1 mice.

We (21) as well as others (2, 3, 6) have demonstrated that encapsulation of doxorubicin inside liposomes reduces the extent of myocardial damage associated with free drug. It is therefore of interest to examine the effect of liposome characteristics on cardiac toxicity. Also, because liposomes accumulate in the liver, it is important to determine whether liver damage occurs on administration of liposomal doxorubicin. The serum levels of CPK, LDH, GOT, and AP enzyme activities were therefore monitored after free doxorubicin was injected and after doxorubicin entrapped in EPC/cholesterol and DSPC/cholesterol liposomes were injected to provide a biochemical index of damage in the liver and heart. These enzymes were chosen since elevated CPK activity in mouse sera is symptomatic of cariomyopathy, and increased AP activity provides an indication of liver toxicity (29). Lactate dehydrogenase and GOT can be derived from either tissue and therefore serve as a secondary screen to corroborate toxicities that can be inferred from the more tissue-specific enzyme activities. Free doxorubicin administered at 20 mg/kg resulted in two- to threefold increases in serum levels of CPK, LDH, and GOT, which persisted over 10 days. Doxorubicin entrapped in EPC/cholesterol liposomes (mean diameter of 160 nm) exhibited transient increases in serum LDH and GOT activities, which returned to control levels by Day 10, while doxorubicin entrapped in DSPC/cholesterol liposomes (mean diameter of 175 nm) did not alter CPK, LDH, and GOT activities when administered at a drug dose of 20 mg/kg (data not shown). All of the doxorubicin treatments studied had no effects on AP serum activities over the 10-day timecourse.

The antitumor efficacy of liposomal doxorubicin as measured in the murine L1210 leukemia model employing EPC/cholesterol (55:45; mol:mol) vesicles with a mean diameter of 180 nm is presented in Table 4. Two points are clear from these results. First, when given at equivalent doses, the liposomal form displays antitumor activity similar to that of the free drug. At doses between 5 and 20 mg/kg, L/F values (the ratio of the ILS of liposomal doxorubicin to that of the free drug) were between 0.85 and 1.03. At the maximum therapeutic doses of free doxorubicin (20 mg/kg), an ILS value of 145% is obtained, while an equivalent dose of liposomal doxorubicin provides a 115% ILS. Second, the maximum ILS value of the liposomal form is greater than that of the free drug because of the reduced toxicity of liposomal doxorubicin. A dose of 30 mg/kg can be administered, resulting in a 190% ILS. This result is illustrated by representative survival curves shown in Fig. 2.

The influence of changes in lipid composition on efficacy for the L1210 leukemia model are also presented in Table 4, where it is shown that at the same dose both the EPC and the DSPC/cholesterol formulations are equally potent as free drug at the 10 and 20 mg/kg doses. For liposomal doxorubicin composed of 100% EPC, the maximum therapeutic dose was similar to that of the free drug (Table 4), as indicated by nontumor-related

Table 4 *Influence of lipid composition on L1210 antitumor activity of liposomal doxorubicin*

Preparation	Drug dose (mg/kg) Dox[a]	Drug dose (mg/kg) Lipid	Survival time (days) 60 Days	Mean	Median	%ILS[b]	L/F[c]
Saline			0/58	10.6	10		
Lipid control		90	0/40	10.7	11	10	
Free Dox	5		0/16	13.8	13	30	
	10		0/46	15.5	15	55	
	20		2/42	28.6	24.5	145	
	25		0/6	17.3	19.0	90	
EPC/C (55:45)	5	15	0/15	12.1	11	10	0.85
	10	30	0/28	15.6	15.5	55	1.03
	20	60	0/18	23.9	21.5	115	0.88
	30	90	1/15	28.9	29.0	190	N/A
	40	120	0/6	13.8	12.0	20	N/A
EPC	10	29	0/6	15.0	15	50	1.00
	20	58	0/6	26.0	25.5	155	1.04
	30	88	0/6	7.2	7.5	−25.0	N/A
DSPC/C (55:45)	10	42	0/6	20.3	17	70	1.13
	20	85	1/6	35	30.5	205	1.27
	30	128	2/6	38.8	32	220	N/A
	50	212	0/6	22.8	23.5	135	N/A
EPC/EPG/C (27.5:27.5:45)	10	30	0/6	16.6	18	80	1.20
	20	60	0/6	28.0	24	140	0.98

[a] Dox, doxorubicin C, cholesterol.
[b] Percentage increase in life span, taken as median survival of treated/median survival of control.
[c] Ratio of median survival time of liposomal-treated animals *versus* animals treated with the equivalent dose of free doxorubicin.

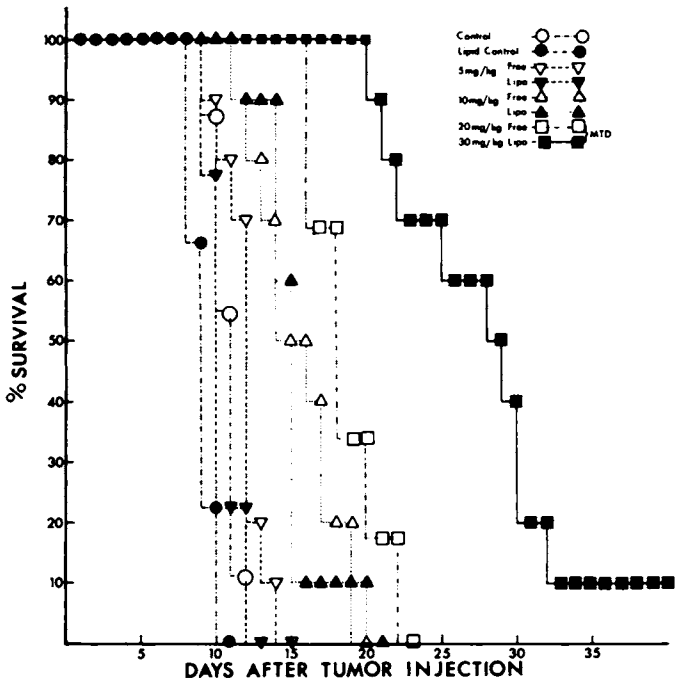

Fig. 2. L1210 antitumor activity of free doxorubicin and 200 nm EPC/cholesterol liposomal doxorubicin. Survival curves are derived from groups of six to 16 DBA/2 mice inoculated i.p. with 1.5×10^6 L1210 cells and subsequently treated i.v. 24 h later. The doses are as indicated in the figure.

Table 5 *Effect of drug to lipid ratio on the toxicity of doxorubicin encapsulated in EPC/cholesterol vesicles*

Doxorubicin was encapsulated in EPC/cholesterol (55:45 mol ratio) vesicles as described in "Materials and Methods."

Dox[a]/total lipid (wt:wt)	LD_{50} (mg/kg body weight)	95% Confidence interval (mg/kg)
0.28:1	57	54–60
0.072:1	45	40–50
0.038:1	39	34–44
0.038:1[b]	38	30–41

[a] Dox, doxorubicin.
[b] Doxorubicin was encapsulated in EPC/cholesterol (55:45 mol ratio) vesicles at a drug-to-lipid ratio of 0.28:1 and subsequently diluted with empty liposomes to achieve a final drug-to-lipid ratio of 0.038:1.

Table 6 *Effect of vesicle size on the toxicity of liposome-encapsulated doxorubicin*

Vesicle-entrapped doxorubicin was prepared as described in "Materials and Methods" at a drug-to-lipid ratio of 0.27 ± 0.04:1 (wt:wt).

Preparation	Mean vesicle diameter ± SD (nm)	LD_{50} (mg/kg body weight)	95% Confidence interval (mg/kg)
EPC/C[a] (55:45)[b]	1400 ± 400	60	56–65
EPC/C (55:45)	160 ± 43	57	54–60
EPC/C (55:45)	106 ± 31	45	40–49
DSPC/C (55:45)	773 ± 140	>200[c]	N/A
DSPC/C (55:45)	175 ± 41	161	148–174

[a] C, cholesterol.
[b] Numbers in parentheses, molar ratios of lipid components.
[c] Only 20% mortality was observed for a dose of 200 mg doxorubicin per kg body weight. Higher doses could not be administered due to the viscosity of the solution.

deaths and a 25% decrease in ILS at a dose of 30 mg/kg. The maximum therapeutic dose of DSPC/cholesterol liposomal doxorubicin (30 mg/kg) results in an ILS value of 220% and two long term (greater than 60 days) survivors out of six. The medium survival times for doxorubicin entrapped in DSPC/cholesterol liposomes are not significantly different from those for the EPC/cholesterol preparations. The maximum therapeutic dose of the DSPC/cholesterol formulation is similar to that of the EPC/cholesterol preparation, even though the LD_{50} of the DSPC-containing formulation is higher. The effect of variations in lipid composition on efficacy was also extended to systems containing the negatively charged phospholipid phosphatidylglycerol. Most liposomal doxorubicin formulations previously employed contain such acidic lipids (see Table 1). As indicated in Table 4, EPC/EPG/cholesterol (27.5:27.5:45; mol:mol:mol) systems exhibit approximately equivalent antitumor activity to that of the free drug at the 10 and 20 mg/kg dose levels.

Role of Drug-to-Lipid Ratio. The versatile encapsulation procedure described here allows the doxorubicin-to-lipid ratio to be varied greatly, and values significantly higher than those used previously (see Table 1) are readily obtained. Such increases in the drug-to-lipid ratio correspond to reduced lipid doses and may be expected to affect the biological activity of entrapped doxorubicin. As indicated in Table 5, decreasing the drug-to-lipid ratio increases the toxicity of liposomal doxorubicin as assessed by dose-response survival studies. Intravenous administration of preparations exhibiting drug-to-lipid ratios of 0.28:1, 0.072:1, and 0.038:1 yielded LD_{50} values of 57, 45, and 39 mg/kg, respectively. It should be noted that this effect was not due to lipid induced toxicity at the lower drug-to-lipid ratios, since no deaths were observed on injection of the highest doses of lipid alone (data not shown). Also, LD_{50} values obtained by diluting samples displaying a drug-to-lipid ratio of 0.28:1 with empty liposomes to achieve a final drug-to-lipid ratio of 0.038:1 are comparable to those observed for vesicles prepared at 0.038 mg doxorubicin/mg lipid. While alterations in the drug-to-lipid ratio affected the toxicity of liposomal doxorubicin, such changes had negligible effects on antitumor potency. Percentage ILS values for animals treated with liposomal doxorubicin preparations varying in drug-to-lipid ratio from 0.28:1 to 0.034:1 at a drug dose of 10 mg/kg were comparable, and L/F values indicated that these systems were equally potent as free drug (data not shown).

Role of Vesicle Size. The above studies employed liposomal doxorubicin systems extruded through filters with 200-nm pore size whose mean diameters range between approximately 160 and 180 nm. The size distribution of liposomal doxorubicin systems is potentially an important characteristic in light of the significant effects vesicle size has on the blood clearance rates of empty liposomes (19). As shown in Table 6, decreasing the mean vesicle diameter of EPC/cholesterol-entrapped doxorubicin preparations from 1400 to 106 nm decreases the LD_{50} value from 60 to 45 mg/kg. This effect is most notable in samples exhibiting mean diameters of 160 and 106 nm, where LD_{50} values of 57 and 45 mg/kg (significantly different as indicated by the 95% confidence intervals), respectively, are obtained. Similar effects are observed for DSPC/cholesterol preparations; decreasing the vesicle size from 773 to 175 nm decreases the LD_{50} in CD1 mice from >200 to 161 mg/kg.

The results presented in Table 7 demonstrate that alterations in vesicle size can have very marked effects on the antitumor activity of doxorubicin in the L1210 leukemia model. For EPC/cholesterol formulations, larger systems prepared from vesicles sized through filters with 1-μm pore size are significantly ($P < 0.05$) less active than free drug, as indicated by the L/F value of 0.67. In contrast, EPC/cholesterol formulations prepared with vesicles sized through 0.1-μm pore size filters are significantly ($P < 0.05$) more active than the free drug (L/F value of 1.94). At a dose of 20 mg/kg, a 30% long-term (greater than 60 days) survival rate and an ILS of 375% are obtained for the

Table 7 *Influence of vesicle size on L1210 antitumor activity of liposomal (PC/C; 55:45) doxorubicin*

Preparation	Drug dose (mg/kg) Dox[a]	Lipid	Survival time (days) 60 Days	Mean	Median	%ILS	L/F%
EPC/C							
1110 nm[b]	20	61	0/6	17	16.5	65	0.67[c]
180 nm	20	60	0/18	23.9	21.5	115	0.88
100 nm	20	68	3/10	45.6	47.5	375	1.94[c]
DSPC/C							
770 nm	10	40	0/10	12.6	13	30	0.87[c]
	30	120	3/10	35.6	25	150	N/A
230 nm	10	42	0/6	20.3	17	70	1.13
	30	128	2/6	38.8	32	220	N/A

[a] Dox, doxorubicin; C, cholesterol.
[b] The mean diameter of the vesicle preparation as estimated by QELS techniques.
[c] Indicates that the difference compared with the same dose of free doxorubicin is significant at $P < 0.05$ level.

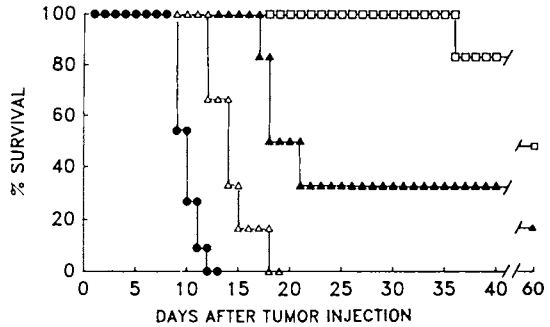

Fig. 3. Survival curves for DBA/2 mice inoculated i.p. with 1.5×10^6 L1210 cells, and were treated i.v. 24 h later with EPC/cholesterol liposomal doxorubicin in which the liposomes were sized through the 100-nm polycarbonate filters before the doxorubicin was encapsulated (see "Materials and Methods"). Survival curves are shown for control animals (●) and animals treated at a dose of 10 mg/kg free drug (△), 10 mg/kg liposomal doxorubicin (▲), and 30 mg/kg liposomal doxorubicin (□).

0.1-μm preparation. Survival curves (Fig. 3) of animals treated with this small liposomal doxorubicin preparation indicate an increase in antitumor activity at a dose of 10 mg/kg. Further, for a dose of 30 mg/kg, a 465% ILS is obtained, with a 50% long-term (greater than 60 days) survival rate.

The trend toward increased antitumor activity as vesicle size is decreased is also observed for DSPC/cholesterol liposomal doxorubicin (see Table 7). At a dose of 10 mg/kg, large (770 nm by QELS) DSPC/cholesterol preparations result in an ILS value of 30%, which is significantly ($P < 0.05$) lower than that obtained for the free drug. At a similar dose, administration of doxorubicin entrapped in DSPC/cholesterol liposomes sized through filters with 200-nm pore size (230 nm by QELS) results in an ILS of 70%, which is equivalent to that of the free drug (L/F value of 1.13). At a dose of 30 mg/kg, ILS values of 150 and 220% are obtained for the 770- and 230-nm systems, respectively. Interestingly, indications of delayed antitumor activity are observed for the 770-nm DSPC/cholesterol systems; similar results are obtained by Storm *et al.* (17) for liposomal doxorubicin preparations composed of saturated lipids. Mice treated with this system at 30 mg/kg display tumor growth (measured as an 8% increase in body weight equivalent to a 1.5-g tumor) comparable to that of the untreated animals at Day 5. Subsequently, no further increase in body weight is observed, and the mean body weight decreases to levels observed before tumor innoculation (data not shown).

Effect of Liposome Encapsulation on the Pharmacokinetics of Doxorubicin. Because vesicle size, lipid composition, and lipid dose can influence the clearance and distribution of liposomes, studies were undertaken to determine the effects of these variables on the pharmacological behavior of doxorubicin. The results in Fig. 4 demonstrate that the tissue-associated doxorubicin levels observed over 24 h post i.v. administration are markedly different for preparations which exhibit widely varying LD_{50} values. Although drug levels in the blood are greatly increased for EPC/cholesterol (LD_{50} = 57 mg/kg) and DSPC/cholesterol (LD_{50} = 161 mg/kg) systems compared to free doxorubicin (LD_{50} = 23 mg/kg), exposure and accumulation of drug over 24 h in tissues normally associated with toxic side-effects (*e.g.*, heart and kidney) are substantially reduced. Conversely, injection of liposomal doxorubicin results in very high levels of drug in the liver and spleen whereas free doxorubicin exhibits tissue levels comparable to those observed in non-reticuloendothelial system-containing tissues.

The distribution of doxorubicin 5 h after injection was selected as a representative timepoint for assessing the roles of vesicle size, lipid composition, and drug-to-lipid ratio in determining the tissue distribution profile in a number of liposome formulations. When doxorubicin encapsulated in EPC/cholesterol (drug-to-lipid ratio = 0.29:1) vesicles is administered, doxorubicin-equivalent levels in the liver and spleen are elevated 5.2- and 25.5-fold, respectively, as compared with levels for the free drug (Table 8). Conversely, drug levels in the heart, lung, and kidney are decreased 3.8-, 1.8-, and 2.1-fold, respectively, comparable to previous observations (4, 7, 9). Omission of cholesterol in EPC vesicle systems results in a total doxorubicin recovery of 11.5%, which is similar to that observed for free drug (Table 8). Drug levels obtained in the liver and spleen for this preparation are lower than those observed for EPC/cholesterol systems. In addition, accumulation of the drug in cardiac tissue is reduced only by a factor of 2.06 for EPC liposomes relative to free drug. Substituting DSPC for EPC decreases drug levels in the heart and kidney while blood levels increase 1.7-fold. Increasing the size of DSPC/cholesterol systems causes an increase in the relative accumulation of doxorubicin in the liver and a slight decrease in the relative accumulation of the drug in the spleen. Negligible amounts of drug are detected in heart tissue for the large DSPC/cholesterol systems, and levels in the lung and kidney are more than 10-fold lower than those observed for free drug. Vesicle systems containing the negatively charged phospholipid EPG exhibit similar doxorubicin equivalent levels in liver, spleen, heart, and kidney tissues, as do liposomes composed of EPC/cholesterol at high drug-to-lipid ratios (Table 8). However, the amount of doxorubicin in lung tissue is increased relative to that of other liposomal doxorubicin preparations examined.

Decreasing the drug-to-lipid ratio in EPC/cholesterol liposomes significantly alters the tissue distribution of doxorubicin equivalents (Table 8). First, total drug recovery is reduced from approximately 54% for a drug-to-lipid ratio of 0.29:1 (wt:wt) to 26% for systems with a drug-to-lipid ratio of 0.037:1 (wt:wt). Second, for the lower drug-to-lipid ratio, drug levels in the liver and spleen are decreased approximately 4-fold, whereas drug levels in kidney, lung, and heart tissues are elevated. The 1.8-fold increase in heart tissue uptake of doxorubicin observed for the lower drug-to-lipid ratio is particularly notable.

The blood clearance behavior for free and liposomal doxorubicin is illustrated in Fig. 5. For a dose of 5 mg/kg doxorubicin, drug levels of 1.61 and 31.5 μg/ml of blood are obtained 5 min after injection for free and liposomal doxorubicin, respectively. Omitting cholesterol from EPC liposome systems causes an increase in the rate of removal of doxorubicin from the blood, approaching that observed for free drug. Blood levels

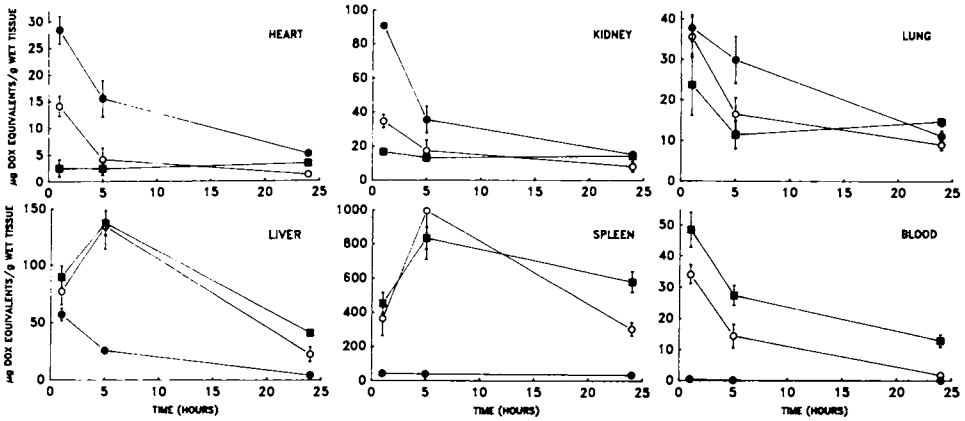

Fig. 4. Kinetic analysis of tissue levels of doxorubicin equivalents in mice subsequent to i.v. administration of 20 mg/kg free dox (●) or doxorubicin encapsulated in 200-nm sized EPC/cholesterol (○) or DSPC/cholesterol (■) at a drug-to-lipid ratio of 0.27:1. Blood levels reflect μg of doxorubicin per ml of blood.

Table 8 *Tissue distribution of doxorubicin equivalents 5 h post-i.v. injection of mice*

Doxorubicin was administered i.v. in all cases at a dose of 20 mg/kg body weight. Doxorubicin/total lipid weight ratios for liposomal doxorubicin were 0.27 ± 0.04 mg/mg lipid. Reported values represent the mean and standard deviation.

Sample	Liver (μg/g)	Spleen (μg/g)	Heart (μg/g)	Lung (μg/g)	Kidney (μg/g)	Blood (μg/ml)	Total recovery (% of administered dose)
Free	25.8 ± 3.7	39.0 ± 8.3	15.5 ± 3.4	29.9 ± 5.8	35.5 ± 7.9	0.09 ± 0.04	10.1
EPC	26.8 ± 1.6	154.1 ± 13.2	7.5 ± 0.6	20.4 ± 0.9	23.9 ± 4.5	1.0 ± 1.0	11.5
EPC/C[a] (55:45)	134.0 ± 20.2	995.1 ± 284.7	4.1 ± 2.2	16.5 ± 4.1	17.3 ± 6.2	14.3 ± 3.8	62.2
EPC/C[b] (55:45)	35.2 ± 2.6	341.9 ± 20.7	7.3 ± 2.2	18.3 ± 2.0	20.5 ± 2.3	25 ± 1.0	26.2
DSPC/C (55:45; 230 nm)	137.5 ± 10.7	832.2 ± 64.8	2.4 ± 1.2	11.4 ± 3.4	13.1 ± 2.1	27.4 ± 3.2	72.7
DSPC/C (55:45, 770 nm)	212.6 ± 12.2	698.3 ± 89.1	<0.5[c]	2.6 ± 1.5	1.9 ± 0.25	10 ± 1.0	68.7
EPC/EPG/C (27.5:27.5:45)	284 ± 28.4	566 ± 51.5	1.25 ± 1.4	30.4 ± 5.6	11.0 ± 2.4	2.0 ± 1.2	69.3

[a] C, cholesterol.
[b] Doxorubicin was encapsulated in EPC/C (55:45) vesicles at a drug-to-lipid ratio of 0.037:1 (wt:wt).
[c] This value reflects minimum detection limits.

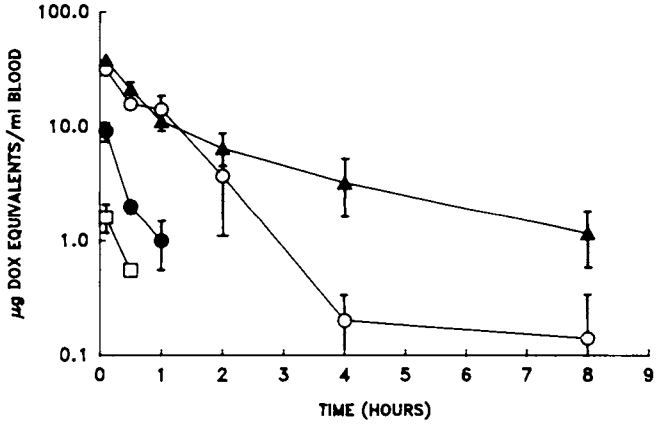

Fig. 5. Blood clearance kinetics for free doxorubicin (□) and doxorubicin encapsulated in EPC (●) and EPC/cholesterol (55:45; mol:mol) (○, ▲) liposomes at a drug-to-lipid weight ratio of 0.27 ± 0.04. Egg PC/cholesterol liposomes exhibited mean diameters of 160 (○) and 106 (▲) nm. Blood samples were collected and analyzed for doxorubicin and its fluorescent metabolites as described in "Materials and Methods." *Error bars*, standard deviation determined from five animals injected i.v. with doxorubicin at a dose of 5 mg/kg.

of 9.2 μg/ml are observed at 5 min for this preparation and decrease to 1 μg/ml within 1 h. Decreasing the liposome size to 106 nm results in doxorubicin blood clearance kinetics that appear comparable to those of larger (>160 nm) liposomal systems over the first 2 h. However, increased blood levels are obtained for the 106 nm preparation at 4 h after drug administration and beyond.

DISCUSSION

Vesicle size, surface charge, lipid composition, and lipid dose are major determinants in the fate of i.v. administered liposomes (19, 30–32). However, the effects of these parameters on the toxicity or efficacy of liposomal doxorubicin have not been well characterized. Progress in this area has been hindered primarily by problems inherent in passive trapping protocols for doxorubicin. Specifically, alterations in one liposome characteristic (such as lipid composition) often result in concomitant changes in other properties (such as liposome size or drug-to-lipid ratio, Refs. 4, 5, 17). Consequently, the importance of individual liposome characteristics in buffering doxorubicin toxicity or altering antitumor efficacy has been difficult to assess. The use of pH gradient-driven uptake to encapsulate doxorubicin in extruded vesicle systems circumvents such complications and therefore provides a significant advance in the ability to address these issues. In addition, this encapsulation protocol exhibits several other pharmaceutically desirable features. First, because efficient trapping is accomplished independent of lipid composition, labile lipids (such as cardiolipin) can be omitted unless dictated by biological response requirements. Second, the simplicity of the ΔpH active entrapment procedure allows doxorubicin to be encapsulated into preformed vesicles immediately prior to use and does not require the removal of free drug. Such a "remote loading" protocol alleviates the possible stability problems related to chemical integrity of the drug and drug retention in the vesicles that are inherent in passive trapping procedures (33).

The results presented here indicate that the changes in lipid composition which result in different *in vivo* stability characteristics markedly affect the acute toxicity of encapsulated doxorubicin. For example, a decrease in LD_{50} values is associated with decreased liposome cholesterol content (Table 3), and doxorubicin encapsulated in EPC liposomes exhibits blood clearance kinetics and tissue distributions approaching those for free drug (Fig. 5 and Table 8). These observations are consistent with results indicating that destabilization of cholesterol-poor liposomes by serum factors leads to release of en-

trapped contents (34–36). LD_{50} values indicate that EPC liposomes are able to significantly buffer the toxicity of doxorubicin. This effect presumably reflects alterations in the distribution of the drug that occur shortly after administration. Encapsulation of doxorubicin in DSPC/cholesterol liposomes causes a dramatic increase in the LD_{50} (161 mg/kg for the systems sized through filters with 200-nm pore size), which is approximately 3- and 8-fold greater than that observed for the EPC/cholesterol systems and free drug, respectively. These LD_{50} values are much higher than those achieved for previous formulations of liposomal doxorubicin (5) and are accompanied by very low drug levels in heart, lung, and kidney tissues (Fig. 4 and Table 8) as well as decreased serum levels of CPK, GOT, and LDH. It is logical to suggest that the DSPC/cholesterol systems are better able to resist serum-induced leakage while in the circulation, a finding that would be consistent with the enhanced stability properties of vesicle systems containing long, saturated acyl-chain components (17, 19).

Interestingly, the acute toxicity of liposomal doxorubicin increases with decreasing drug-to-lipid ratio. This result may have general implications in light of similar effects seen for liposomal amphotericin B (37). For a constant doxorubicin dose, decreasing the drug-to-lipid ratio results in an increased lipid dose to the animal. Such increases may be expected to lead to increased longevity of liposomes in the circulation (31, 32). Thus, the increased toxicity observed for low doxorubicin-to-lipid ratios likely results from increased leakage caused by extended exposure to blood components and subsequent accumulation of free drug in tissues associated with doxorubicin toxicity. This interpretation is supported by the elevated levels of doxorubicin in the blood and heart tissue observed with systems having low drug-to-lipid ratios (Table 8). Similar arguments would also appear consistent with the relationship between the circulation longevity of small (100 nm) liposomal systems (Fig. 4) and toxicity.

As previously indicated, a number of investigations have reported enhanced antitumor activities of liposomal doxorubicin preparations. The increase in the therapeutic index of doxorubicin obtained when it is encapsulated in liposomes is primarily due to reduced toxicity rather than increased antitumor potency. This result is illustrated in this report (Table 4 and Fig. 2) for the 170-nm EPC/cholesterol liposomal doxorubicin formulation, which is as efficacious as the free drug when administered at equivalent doses. As observed for other liposomal doxorubicin preparations (4, 5, 7, 16), the maximum therapeutic dose of the liposomal drug is greater than free drug, thus providing an enhanced antitumor effect.

The results of the present study point out that an important feature related to obtaining optimal efficacy concerns liposome size. For the L1210 tumor model employed here, EPC/cholesterol liposomal doxorubicin with size distributions in the range of 100 nm clearly exhibits enhanced antitumor activity over free drug, whereas large (approximately 1 μm) systems are less effective (Table 8). Gabizon and coworkers (4, 12, 13) also observed enhanced efficacy of small systems employing an SUV liposomal doxorubicin preparation (90 nm) for the treatment of J6456 lymphoma in mice. They concluded that the SUV doxorubicin formulation was more efficacious than an MLV formulation due to increased accessibility to the parenchymal area of the liver where the tumor cells seed. It is important to note that in this earlier study (4) a single dose of the MLV preparation was compared with a multiple dose of the SUV system. The extended circulation half-life of small vesicles (100 nm; Fig. 5) as well as the increased accessibility to other tissues (38, 39) may be important in enhancing the therapeutic activity of this liposomal drug carrier system.

The data presented here indicating that large (1 μm) systems are less active than the free drug contrast with reports indicating that large liposomal doxorubicin systems display antitumor activity similar to that of the free drug (3, 4). Such differences may reflect either increased *in vivo* stability of the preparations employed here or the 5- to 10-fold greater drug-to-lipid ratio. An additional indication of size-dependent effects is seen for large (770 nm) DSPC/cholesterol liposomal doxorubicin preparations in which a delayed antitumor effect was observed. A similar effect was noted by Storm *et al.* (17), who showed a delay in antitumor activity of liposomal doxorubicin formulations composed of DPPC/DPPG/cholesterol (820 nm diameter) as compared with a preparation composed of EPC/bovine brain phosphatidylserine/cholesterol (270 nm diameter). The results presented here suggest that these differences in antitumor activity may be related primarily to liposome size. For example, smaller (230 nm diameter) DSPC/cholesterol systems are more effective than larger (770 nm diameter) systems (Table 7).

In summary, encapsulation of doxorubicin in liposomes employing ΔpH-dependent uptake procedures results in trapping efficiencies approaching 100% for drug-to-lipid ratios as high as 0.3:1 (wt:wt) and excellent drug retention properties. In addition, this encapsulation technique is relatively independent of variables such as lipid composition, vesicle size and drug-to-lipid ratio. The flexibility of the liposome generation and loading procedures used here have allowed new insights to be gained into factors influencing drug toxicity. Specifically, whereas inclusion of negatively charged lipids does not appear to greatly influence LD_{50} values, changes in liposomal properties that would be expected to result in more stable drug retention in the circulation, or reduced residence times in the circulation, can greatly decrease acute toxicity. The antitumor activity of liposomal doxorubicin is relatively independent of lipid composition and drug-to-lipid ratio. Higher doses of the less toxic preparations can be administered, leading to enhanced efficacy. However, these studies clearly demonstrate that antitumor potency is dependent on vesicle size. These findings establish that the biological activity of liposomal doxorubicin preparations can be markedly sensitive to the physical characteristics of the formulation. Elucidation of pivotal factors through further in-depth studies in areas such as pharmacokinetics, myelosuppression, and tumor specificity may be expected to lead to a more detailed understanding of the mechanism of action of liposomal doxorubicin and development of optimum preparations appropriate to particular applications.

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