Liposomal Vincristine Preparations Which Exhibit Decreased Drug Toxicity and Increased Activity against Murine L1210 and P388 Tumors

Lawrence D. Mayer, Marcel B. Bally, Helen Loughrey, et al.

Cancer Res 1990;50:575-579.

[CANCER RESEARCH 50, 575-579, February 1, 1990]

Liposomal Vincristine Preparations Which Exhibit Decreased Drug Toxicity and Increased Activity against Murine L1210 and P388 Tumors[1]

Lawrence D. Mayer, Marcel B. Bally, Helen Loughrey, Dana Masin, and Pieter R. Cullis

Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1W5 [L. D. M., M. B. B., H. L., P. R. C.], and The Canadian Liposome Co., Ltd., North Vancouver, British Columbia V7M 1A5 [L. D. M., M. B. B., D. M., P. R. C.], Canada

ABSTRACT

The toxicity and antitumor activity of liposomal vincristine preparations have been examined. Vincristine was encapsulated inside egg phosphatidylcholine (EPC)/cholesterol (55/45, mol/mol) and distearoylphosphatidylcholine (DSPC)/cholesterol (55/45, mol/mol) vesicles utilizing transmembrane pH gradient (inside acidic) drug uptake processes. Trapping efficiencies approaching 100% were achieved for this procedure using drug:lipid ratios as high as 0.2:1 (w/w). Although both EPC/cholesterol and DSPC/cholesterol liposomal systems yielded high trapping efficiencies, DSPC/cholesterol vesicles exhibited superior drug retention properties. This ability to retain entrapped vincristine was related to maintenance of the transmembrane pH gradient as well as the membrane permeability properties. Thirty-day dose-response survival studies in mice indicated that vincristine encapsulated in DSPC/cholesterol liposomes was less toxic than free drug. The 50% lethal dose of 1.9 mg/kg in CD-1 mice observed for free vincristine increased to 4.8 mg/kg upon administration of the drug in liposomal form. Liposome encapsulation of vincristine also enhanced the antitumor activity against murine P388 and L1210 lymphocytic leukemia models. This resulted from increased efficacy for liposomal vincristine at doses equal to free drug (liposomal/free drug median survival times > 1.0) as well as the ability to administer increased doses of liposomal vincristine. The combined effects of decreased toxicity and increased antitumor efficacy of liposomal vincristine over free drug suggest significant clinical utility of appropriate liposomal vincristine systems.

INTRODUCTION

Liposome encapsulation of various antineoplastic agents has been shown to decrease drug-induced toxic side effects while maintaining or, in some instances, increasing antitumor activity (1-12). Reduction of toxicity results from the ability of liposomes to decrease drug exposure, and subsequent damage, to susceptible tissues (4, 13-16). The mechanism of the antitumor activity of entrapped drugs is less well understood but may result from the capacity of liposomes to slowly release encapsulated drug into the circulation (17, 18) or alternatively passive targeting of liposomes and their contents to tumor sites (6, 10, 11, 19, 20). Most of these studies have been performed on liposomal doxorubicin which is currently under clinical investigation (21, 22) Antitumor drugs of the *Vinca* alkaloid class (vincristine and vinblastine) are also important agents in cancer chemotherapy; however, only preliminary work has been done to apply liposome technology to these agents (23, 24). In addition, these investigations have not demonstrated that liposome encapsulation of vincristine provides a therapeutic advantage (24).

Vincristine exhibits low solubility in aqueous solutions at physiological pH and relatively high permeability to membranes. These two features in addition to constraints inherent in conventional drug entrapment procedures (25) has limited the development of stable liposomal vincristine formulations. We have previously shown that transmembrane pH gradients (inside acidic) can be utilized to circumvent such complications for lipophilic, cationic drugs such as doxorubicin (26), dibucaine (27), and dopamine (28). This process yields stable liposome systems with trapping efficiencies approaching 100% and very high levels of entrapped drug in a manner which is relatively independent of liposome size and lipid composition. We describe here the development of liposomal vincristine preparations which use transmembrane pH gradients to obtain trapping efficiencies in excess of 98% and drug:lipid ratios as high as 0.2:1 (w/w). It is shown that, compared to free vincristine, these systems display reduced toxicity and increased antitumor activity in mouse models.

MATERIALS AND METHODS

"Oncovin" (vincristine sulfate) was obtained from the Cancer Control Agency (Vancouver, British Columbia, Canada). EPC[2] and DSPC were purchased from Avanti Polar Lipids and were greater than 99% pure. Cholesterol and all salts were obtained from Sigma Chemical Co. (St. Louis, MO). DBA/2J and CD-1 mice (6-8 weeks old) were purchased from Jackson Laboratory Animals and Charles River, respectively. EPC/cholesterol or DSPC/cholesterol lipid films were prepared by vacuum evaporation from a $CHCl_3$ solution. Lipids were then hydrated in 300 mM citric acid (pH 4.0) by vortex mixing, using a lipid/buffer ratio of 100 mg/ml. The multilamellar vesicles were frozen and thawed 5 times (29), and then extruded 10 times through 200 nm pore size polycarbonate filters (30), using a lipid extrusion device obtained from Lipex Biomembranes (Vancouver). Production of the DSPC/cholesterol samples required heating the sample and extruder to 60°C during extrusion. Mean vesicle diameters determined by quasielastic light scattering (using a Nicomp 270 particle sizer) were 170 and 220 nm for the EPC/cholesterol and DSPC/cholesterol liposomes, respectively. Vesicles (100 mg/ml) were added to the Oncovin solution (1 mg vincristine/ml) to achieve the indicated drug/lipid ratios. The pH of the sample was then raised to pH 7.0-7.2 with 1 M Na_2HPO_4. These solutions were then heated at 60°C for 10 min unless otherwise indicated. Vincristine entrapment was determined by column chromatography techniques (27) using A_{297} (in ethanol/H_2O, 8/2) and A_{815} (27) spectroscopic assays for quantitation of vincristine and lipid, respectively. In the case of large unilamellar vesicle systems using inorganic phosphate, initial drug/lipid ratios were determined prior to the alkalinization step.

Drug retention was measured at 21°C at a drug concentration of 0.5 mg/ml. Aliquots were assayed at the indicated times after passage of the liposomes over Sephadex G-50 columns to remove free from vesicle-associated drug. Residual pH gradients were determined by monitoring the transmembrane distribution of [^{14}C]methylamine (0.5 μCi/ml) added after vincristine encapsulation as described previously (27).

Dose-response survival studies were performed by injecting the indicated amounts of free or liposome-encapsulated vincristine via a lateral tail vein to female DBA/2J or CD-1 mice (18-22 g, 10 mice/group) in 0.2 ml with sterile saline as the diluent. Mortality and average body weight were monitored over 30 days. LD_{50} and 95% confidence interval values were determined using logit dose-response analysis as described by Williams (31). Calculations were performed by using

Received 6/6/89; revised 8/21/89, 10/18/89; accepted 10/30/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Cancer Institute of Canada and The Liposome Co., Inc. (Princeton, NJ). P. R. C. is a Canadian Medical Research Council scientist.

[2] The abbreviations used are: EPC, egg phosphatidylcholine; DSPC, distearoylphosphatidylcholine; ILS, increase in life span; LD_{50}, 50% lethal dose.

generalized linear modeling. Peripheral WBC were obtained by collecting the blood of DBA/2J mice (4 mice/group) from the carotid artery into "Microtainer" tubes containing EDTA beads, diluting the blood 10-fold with 2% acetic acid, and counting the WBC with a hemocytometer. Bone marrow WBC were obtained by flushing the tibia and femur from one leg of DBA/2J mice (4 mice/group) with 10 ml of isotonic minimal essential media, creating a cell suspension by passing the bone marrow 3 times through a 22.5-gauge needle and counting the WBC with a hemocytometer after suspending the pelleted cells ($500 \times g$) in minimal essential media/4% acetic acid (50:50).

The antitumor activity of free and liposomal vincristine was assessed by using L1210 lymphocytic and P388 leukemia models. DBA/2J mice (6 mice/group) were given injections i.p. of 1×10^6 L1210 cells or 1×10^5 P388 cells derived from the ascites fluid of a previously infected mouse. The indicated doses of saline, empty liposomes, free vincristine, and liposomal vincristine were administered i.v. at the indicated times after tumor inoculation and animal weights as well as mortality rates were monitored. ILS values were calculated from median survival times. Mean and medium survival times and statistical significance of the results were determined by using a 2-tailed Wilcoxon's ranking test (randomized two group design).

RESULTS

Characterization of ΔpH-dependent Vincristine Entrapment in Liposomes. Incubation of lipophilic cationic drugs with vesicles exhibiting transmembrane K^+ or H^+ gradients can result in accumulation of these agents into the interior of the liposome (26–28, 32, 33). These uptake processes are sensitive to variables such as internal buffering capacity, lipid composition, and temperature (27). We have previously demonstrated (26) that elevated temperatures are required to obtain efficient encapsulation of doxorubicin inside EPC/cholesterol vesicles exhibiting a transmembrane pH gradient (inside acidic). In contrast, Fig. 1 shows that vincristine rapidly accumulates inside EPC/cholesterol (55/45, mol/mol) vesicles exhibiting a ΔpH of 3.0 units at 21°C, leading to trapping efficiencies in excess of 90% within 30 min. DSPC/cholesterol (55/45, mol/mol) vesicles exhibiting the same transmembrane pH gradient, however, sequester only 17% of the available vincristine over 1 h at 21°C (Fig. 1). This value can be increased to greater than 98% for 10-min incubations by increasing the temperature to 60°C (Fig. 1). It should be noted that trapping efficiencies in excess of 95% can be obtained for drug/lipid ratios as high as 0.2:1 (w/w). In addition, vincristine encapsulation does not alter vesicle size as determined by quasielastic light scattering (data not shown).

The retention of vincristine in DSPC/cholesterol and EPC/cholesterol liposomes was monitored to determine the stability of the vesicle systems subsequent to drug entrapment. No detectable vincristine release is observed over 24 h at 21°C for liposomes composed of DSPC/cholesterol (55/45, mol/mol; Fig. 2*A*). EPC/cholesterol (55/45, mol/mol) vesicles, however, release approximately 40% of entrapped vincristine after 24 h.

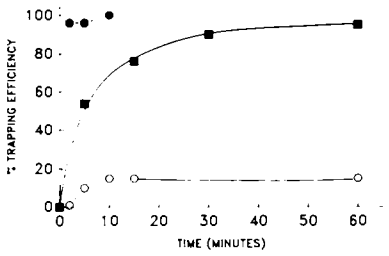

Fig. 1. Uptake of vincristine into DSPC/cholesterol (●, ○) and EPC/cholesterol (■) vesicles at 21°C (○, ■) and 60°C (●). Incubation conditions and determination of free and liposome-associated vincristine were as described in "Materials and Methods."

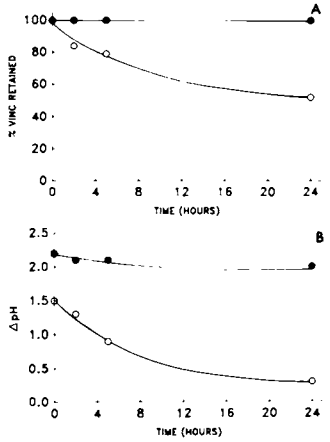

Fig. 2. *A*, vincristine release from DSPC/cholesterol (●) and EPC/cholesterol (○) vesicles subsequent to ΔpH-dependent drug accumulation. Vincristine was encapsulated at a drug/lipid ratio (w/w) of 0.19 ± 0.01/1 (SD). Liposomes were incubated at 21°C and at the indicated times vesicle-associated drug was determined as described in "Materials and Methods." *B*, transmembrane pH gradient dissipation at 21°C in DSPC/cholesterol (●) and EPC/cholesterol (○) vesicles subsequent to vincristine encapsulation at a drug/lipid ratio (w/w) of 0.19± 0.01/1. Incubation conditions were identical to those described in *A*. Transmembrane pH gradients were determined by using [^{14}C]methylamine as described in "Materials and Methods."

The ability of vesicles to retain vincristine appears related to the ability of the liposomes to maintain a large (greater than 2.0 units) pH gradient. DSPC/cholesterol vesicles exhibit a transmembrane pH gradient in excess of 2.0 units, whereas the pH gradient across EPC/cholesterol systems decreases from 1.5 units to less than 0.5 unit over the 24-h time course (Fig. 2*B*). Also, the rate of transmembrane pH gradient collapse is comparable to vincristine release in EPC/cholesterol vesicles (compare Fig. 2*A* and 2*B*). Further, collapsing the pH gradient of DSPC/cholesterol systems by lowering the external pH results in 27% vincristine release over 24 h at room temperature and 92% release over 1 h at 60°C (data not shown). No decrease in the ΔpH is observed under the conditions used in the absence of vincristine for DSPC/cholesterol or EPC/cholesterol liposomes (data not shown).

Toxicity of Liposomal Vincristine. Due to the poor retention of vincristine in EPC/cholesterol preparations (Fig. 2), the *in vivo* activity of liposomal vincristine was studied by utilizing DSPC/cholesterol vesicles. The 30-day dose-response survival curves for free and liposomal vincristine in DBA/2J mice are shown in Fig. 3*A*. For free vincristine, a sharp mortality dose dependence is observed between 2.0 and 3.0 mg/kg yielding a LD_{50} of 2.5 mg/kg (Table 1). This compares favorably with published LD_{50} values (23). Encapsulating vincristine in DSPC/cholesterol vesicles results in an increase of the LD_{50} to 4.2 mg/kg (Table 1). It should be noted that the majority of deaths occurred within 14 days of drug administration (the majority of deaths occurring on day 5 to day 10), and surviving mice which had suffered weight loss within this time period (up to 30% weight loss) regained weight, approaching control values by day 30. The onset of toxicity is also revealed in the weight loss of animals 7 (nadir value) days after receiving free and liposomal vincristine (Fig. 3*B*). Although the curves do not exhibit as sharp a dose dependence as do the mortality curves, the ability of liposome encapsulation to displace the drug dose required to induce weight loss also indicates the reduced toxicity of liposomal vincristine. No distinct differences in weight loss characteristics and the time frame of death could be ascertained between free and liposomal vincristine at the respective maximum tolerated doses. Similar trends in toxicity behavior are

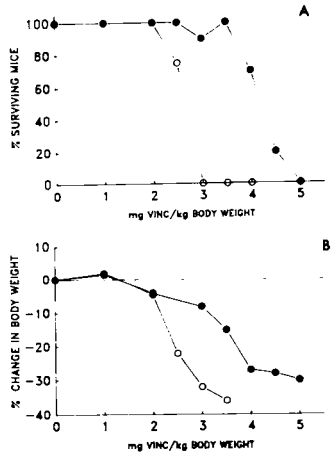

Fig. 3. *A*, 30-day dose-response survival curves for free vincristine (○) and vincristine entrapped inside DSPC/cholesterol liposomes (●) administered i.v. to female DBA/2J mice. *B*, dose-response curve for the percentage of change in body weight of female DBA/2J mice 7 days after i.v. administration of free (○) or DSPC/cholesterol-encapsulated (●) vincristine.

Table 1 *Toxicity of free and liposomal vincristine*

Free or liposomal vincristine was administered i.v. as described in "Materials and Methods," and survival was monitored for 30 days. Vincristine was encapsulated in DSPC/cholesterol liposomes at a drug/lipid ratio (w/w) of 0.17–0.19:1, as described in "Materials and Methods."

Sample	Mouse strain	LD_{50} (mg/kg)	95% confidence interval (mg/kg)
Free vincristine	DBA/2J	2.5	2.4–2.7
Liposomal vincristine	DBA/2J	4.2	3.8–4.4
Free vincristine	CD-1	1.9	1.4–2.1
Liposomal vincristine	CD-1	4.8	4.3–5.4

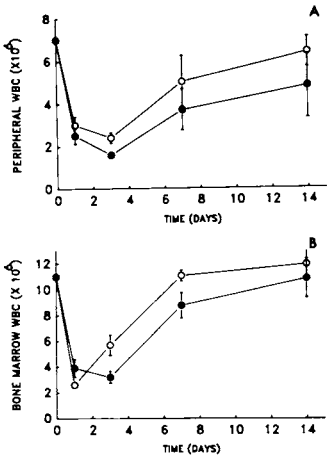

Fig. 4. *A*, peripheral WBC/1.0 ml of blood in DBA/2J mice following i.v. administration of free (○) and DSPC/cholesterol-entrapped (●) vincristine at a drug dose of 1.5 mg/kg. *B*, bone marrow WBC obtained from the femur and tibia of DBA/2J mice following i.v. administration of free (○) and DSPC/cholesterol-entrapped (●) vincristine at a drug dose of 1.5 mg/kg. *Points*, mean values obtained from 4 animals; *bars*, SD.

obtained with CD-1 mice (Table 1).

The WBC suppressive characteristics of free and liposomal vincristine are shown in Fig. 4. The peripheral and bone marrow WBC respond similarly to free and liposomal vincristine at a dose of 1.5 mg/kg. The drug-induced decrease in WBC population is observed first in the bone marrow, followed by a decrease in the peripheral count with nadirs occurring at approximately days 1 and 3, respectively. The extent of peripheral WBC suppression increases slightly when vincristine is administered in liposomal form. Nadir values for peripheral counts are 2.4×10^6 and 1.7×10^6 for free and liposomal drug, respectively (Fig. 4*A*). Nadirs for bone marrow WBC suppression are comparable for free and liposomal vincristine, ranging from 2.5 to 3.0×10^6 cells. Both peripheral and bone marrow studies indicate that encapsulation of vincristine inside liposomes prolongs the suppression of WBC compared to free drug.

Antitumor Activity of Free and Liposomal Vincristine. Free vincristine increases the survival time of mice infected i.p. with P388 cells in a dose-dependent manner (Table 2). Drug doses i.v. of 1.0 and 2.0 mg/kg result in ILS values of 10.7 and 28.6%, respectively ($P < 0.05$ and 0.01, respectively). Administration of liposomal vincristine results in increased antitumor activity at drug doses of 1.0 and 2.0 mg/kg, yielding ILS values of 14.3 and 57.1%, respectively. The liposomal/free median survival time values of 1.14 and 1.22 (the latter of which is statistically significant; $P < 0.01$) indicate that vincristine encapsulated inside DSPC/cholesterol vesicles is more efficacious than free drug at equal doses. In addition, liposomal vincristine is less toxic than free drug, as shown by the reduced weight loss of mice 8 days after treatment. Due to this reduced toxicity, a vincristine dose of 3.0 mg/kg can be given in liposome encapsulated form, resulting in an ILS value of 96.4%.

Subsequent studies investigated the efficacy of free and liposomal vincristine in the L1210 ascites tumor model. Administration of free vincristine 24 h after i.p. inoculation of L1210 cells does not result in increased survival. Free vincristine doses of 0.5 and 1.0 mg/kg result in median survival times which are similar to those for control animals (Table 3). Increasing the dose of free drug to 2.0 mg/kg decreases the median survival time (Table 3). In contrast to free drug, liposomal vincristine displays a dose-dependent antitumor activity against the L1210 model. Increasing the drug dose (single injection) from 0.5 mg/kg to 2.0 mg/kg increases the ILS value from 16.7 to 72.2% (Table 3). The decreased acute toxicity of liposomal vincristine allows the administration of higher drug doses and results in a maximum ILS of 116% at 3.0 mg vincristine/kg. At 4.0 mg/kg, drug-induced toxicity is observed, indicating that the maximum tolerated dose of liposomal vincristine for this tumor model is 3.0 mg/kg.

Multiple dose regimens in the L1210 tumor model were also investigated in attempts to increase the therapeutic activity of free vincristine as well as to compare the efficacy of liposomal drug with single-dose treatment schedules. Extending the time of free vincristine administration by using multiple-injection

Table 2 *P388 antitumor activity of free and liposomal vincristine in DBA/2J mice*

Mice were given injections i.v. of the indicated samples 24 h post-i.p. injection of 1×10^5 P388 cells isolated from the ascites fluid of a previously infected DBA/2J mouse. Vincristine was encapsulated inside DSPC/cholesterol vesicles at a drug:lipid ratio of 0.17:1 (w/w) as described in "Materials and Methods."

Sample	Dose (mg/kg) Drug	Dose (mg/kg) Lipid	% of wt change on day 8	Survival time (days) 40-day	Mean	Median	% of ILS[a]	L/F[b]
Saline control			5.7	0/6	14.0	14.0		
Free vincristine	1.0		−1.3	0/6	15.3	15.5	10.7[c]	
	2.0		−9.5	0/6	17.8	18.0	28.6[d]	
Liposomal vincristine	1.0	5.8	0.0	0/6	16.8	16.0	14.3[d]	1.14
	2.0	11.6	−2.5	0/6	22.0	22.0	57.1[d]	1.22[c]
	3.0	17.6	−16.4	0/6	26.8	27.5	96.4[d]	

[a] Percentage of ILS values were determined from median survival times comparing treated and saline control groups.

[b] L/F, liposomal/free median survival time; values were determined by dividing the median survival time of the liposomal group by the median survival time of mice administered the equivalent dose of free vincristine.

[c] Significant at the $P < 0.05$ level.

[d] Significant at the $P < 0.01$ level.

Table 3 *L1210 antitumor activity of free and liposomal vincristine in DBA/2J mice*

Sample	Dose (mg/kg) Drug	Lipid	Survival time (days) 40-day	Mean	Median	ILS (%)	L/F[a]
Saline control			0/18	9.5	9.0		
Empty liposomes		30	0/12	9.7	9.0	0	
Free vincristine[b]	0.5		0/12	9.5	9.5	5	
	1.0		0/12	9.3	9.0	0	
	2.0		0/12	7.7	8.0	−11.2	
Liposomal vincristine[b]	0.5	2.9	0/16	10.8	10.5	16.7	1.11[c]
	1.0	5.8	0/22	14.5	14.0	55.5[d]	1.55[c]
	2.0	11.6	0/22	15.8	15.5	72.2[d]	1.94[c]
	3.0	17.6	0/16	20.0	19.5	116.6[d]	
	4.0	23.2	0/10	8.5	8.0	−11.2	
Saline control[e]			0/6	10.6	11		
Free vincristine[e]	0.5		0/6	12.0	12.0	9.0	
	0.75		0/6	12.0	12.0	9.0	
	1.0		0/6	11.8	12.0	9.0	
Liposomal vincristine[e]	0.5	2.9	0/6	14.2	14	27.3[d]	1.17
	1.0	5.8	1/6	26.0	21.0	91.0[d]	1.75[c]
	1.5	8.8	0/6	17.2	18.0	63.6[d]	
Saline control[f]			0/6	11.1	11		
Free vincristine[f]	0.75		0/6	13.2	13.0	18.2[d]	
	1.0		0/6	12.5	13	18.2	
Liposomal vincrinstine[f]	0.75	4.4	0/6	17.0	17.0	54.5[d]	1.31[c]
	1.0	5.8	1/6	25.0	22.5	104.5[d]	1.73[c]
	1.5	8.8	2/6	26.5	21.0	90.9[d]	

[a] L/F, liposomal/free median survival time.
[b] DBA/2J mice were given injections i.v. of the indicated doses of drug and/or lipid 24 h post-i.p. injection of 1×10^6 L1210 cells/mouse.
[c] Significant at the $P < 0.05$ level.
[d] Significantly different from control ($P < 0.05$).
[e] DBA/2J mice were given injections i.v. of the indicated doses of drug and/or lipid on days 1, 3, and 5 post-i.p. injection of 1×10^6 L1210 cells/mouse.
[f] DBA/2J mice were given injections i.v. of the indicated doses of drug and lipid on days 1, 5, and 9 post-i.p. injection of 1×10^6 L1210 cells/mouse.

protocols increases the antitumor activity, resulting in a maximum ILS value of 18.2% (which is significantly greater than control groups, $P < 0.05$) for a dose of 0.75 mg drug/kg given on days 1, 5, and 9 (Table 3; Fig. 5). Injection of liposomal vincristine on days 1, 3, and 5 or 1, 5, and 9 does not increase the maximum ILS (91 and 104.5%, respectively; calculated from median survival times) over that obtained for a single-dose regimen. However, multiple-dose treatment schedules of liposomal vincristine do increase the 40-day survival rate. This effect is most apparent for liposomal vincristine administered on days 1, 5, 9, where 16.7 and 33.3% of the mice survive for 40 days for drug doses of 1.0 and 1.5 mg/kg/injection, respectively (Table 3). As observed for the single-injection schedule, liposomal vincristine is significantly more efficacious than free drug at doses of 0.75 and 1.0 mg/kg (Table 3; Fig. 5).

DISCUSSION

The use of transmembrane pH gradients (inside acidic) to accumulate vincristine inside liposomes can result in efficient and stable drug entrapment. This is reflected by trapping efficiencies approaching 100% in combination with drug/lipid ratios in excess of 200-fold greater than achieved by previous techniques. Drug retention is enhanced for liposomes exhibiting reduced permeability properties and stable transmembrane pH gradients following uptake. In this context, it is interesting to note that drug release and pH gradient dissipation in EPC/cholesterol liposomes occur much faster for vincristine (Fig. 2)

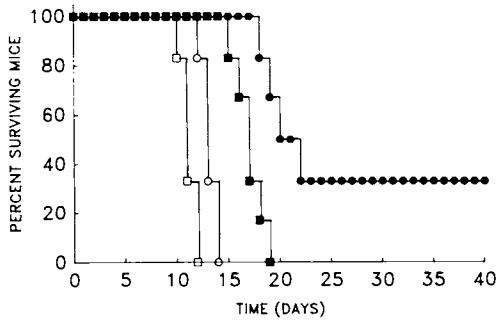

Fig. 5. Survival curves for DBA/2J mice inoculated i.p. on day zero with 1×10^6 L1210 cells and given injections i.v. on days 1, 5, and 9 with saline (□), free vincristine at 0.75 mg/kg (○), liposomal vincristine at 0.75 mg/kg (■), and liposomal vincristine (DSPC/cholesterol) at 1.5 mg/kg (●).

than previously observed for doxorubicin (27). These results are consistent with the rapid uptake of vincristine into EPC/cholesterol vesicles at room temperature (Fig. 1), whereas elevated temperatures are required for doxorubicin accumulation (27). These findings indicate that vincristine is more membrane permeable than doxorubicin. It is apparent that, for both agents, drug entrapment and *in vitro* retention are affected by the magnitude of the ΔpH.

Vincristine entrapped in stable (DSPC/cholesterol) liposome systems results in reduced toxicity as indicated by dose-response survival and weight loss curves. This contrasts with previous observations (24), where liposomal forms of vincristine provided no protective effect in terms of toxicity. Although such differences are not fully understood, this discrepancy may be due to the lack of drug retention in previous liposomal vincristine systems. This interpretation is supported by previous studies with liposomal doxorubicin (26), where stable formulations exhibited decreased toxicity and decreased exposure of the drug to susceptible tissues. It should be noted, however, that the correlation between *in vitro* and *in vivo* drug release properties is not fully resolved. An interesting finding is that liposomal vincristine displays equal or slightly increased suppression of peripheral and bone marrow WBC compared to free drug at equivalent doses. This effect may result from the extended exposure of these cells to vincristine caused by an increase in the circulation time of liposomal drugs (26) or alternatively, from the ability of certain WBC types to phagocytose or endocytose liposomes (34, 35).

In addition to reducing acute toxicity, entrapment of vincristine inside DSPC/cholesterol liposomes by the pH gradient-driven process increases the antitumor efficacy over free drug. In the P388 tumor model, administration of vincristine in liposomal form increases the maximum ILS from 28.6% for free drug to 96.4%. This effect is obtained as a result of enhanced therapeutic activity over free vincristine at equal doses as well as an ability to inject more drug. A more dramatic increase of antitumor activity is seen in the L1210 model, where liposomal/free median survival time values as high as 1.75 are achieved with maximum ILS values of 116%. Furthermore, appropriate dosing schedules of liposomal vincristine results in mice which survive for 40 days under conditions where administration of free drug provides minimal extension of survival time.

The mechanism by which enhanced antitumor activity is obtained for liposomal vincristine is of interest. Previous *in vitro* (36) and *in vivo* (37) studies indicate that increased vincristine efficacy correlates well with increased duration and concentration of drug in the circulation. The results obtained

here are consistent with this interpretation, where extending drug administration from single to multiple dose treatment schedules in the L1210 model increases ILS values and the number of 40-day survivors for free and liposomal vincristine groups, respectively. The ability of liposomes to increase the circulation time of encapsulated agents (14, 38) is likely to result in extended release properties, thereby enhancing the therapeutic activity of the drug. This interpretation is consistent with results from clinical studies which indicated that enhanced response rates could be achieved by administering vincristine as an infusion rather than a bolus injection (39). Interestingly, the increased toxicity observed for infused vincristine (40) contrasts the results here which demonstrate that liposomal vincristine is less toxic than single- or multiple-dose regimens of free drug. As an alternate mechanism for the increased antitumor activity of vesicle-entrapped vincristine, liposomes may preferentially deliver their contents to the tumor site as demonstrated previously for vesicles containing imaging agents such as ^{99}Tc and ^{111}In (19, 20). Such "passive" targeting could lead to increased exposure of vincristine to the tumor and consequently, increased efficacy.

In summary, liposomes encapsulation of vincristine utilizing transmembrane pH gradients provides an efficient means for achieving well-defined vesicle preparations with high drug/lipid ratios and stable drug retention. More importantly, these systems display reduced toxicity and increased antitumor activity compared to free vincristine. The implications of these properties are that such liposomal vincristine preparations will provide a significant therapeutic advantage in clinical applications. Further studies on the pharmacological and neurotoxic characteristics of liposomal vincristine systems may result in a more comprehensive basis for predicting their potential utility.

REFERENCES

1. Gregoriadis, G., and Neerunjun, E. D. Treatment of tumour bearing mice with liposome-entrapped actinomycin D prolongs their survival. Res. Commun. Chem. Pathol. Pharmacol., *10:* 351–362, 1975.
2. Forssen, E. A., and Tokes, Z. A. Use of anionic liposomes for the reduction of chronic doxorubicin-induced cardiotoxicity. Proc. Natl. Acad. Sci. USA, *78:* 1873–1877, 1981.
3. Rahman, A., More, N., and Schein, P. S. Doxorubicin-induced chronic cardiotoxicity and its protection by liposomal administration. Cancer Res., *42:* 1817–1825, 1982.
4. Gabison, A., Dagan, A., Goren, D., Barenholz, Y., and Fuks, Z. Liposomes as *in vivo* carriers of Adriamycin: reduced cardiac uptake and preserved antitumor activity in mice. Cancer Res., *42:* 4734–4739, 1982.
5. Herman, E. H., Rahman, A., Ferrans, V. J., Vicks, J. A., and Schein, P. S. Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation. Cancer Res., *43:* 5427–5432, 1983.
6. Gabizon, A., Goren, D., Fuks, Z., Barenholz, Y., Dagan, A., and Meshorer, A. Enhancement of Adriamycin delivery to liver metastatic cells with increased tumoricidal effect using liposomes as drug carriers. Cancer Res., *43:* 4730–4735, 1983.
7. Mayhew, E., and Rustum, Y. M. The use of liposomes as carriers of therapeutic agents. Prog. Clin. Biol. Res., *172B:* 301–310, 1985.
8. Hunt, C. A., Rustum, Y. M., Mayhew, E., and Papahadjopoulos, D. Retention of cytosine arabinoside in mouse lung following intravenous administration in liposomes of different size. Drug Metab. Dispos., *7:* 124–128, 1979.
9. Kobayashi, T., Kataoka, T., Tsukagoshi, S., and Sakurai, Y. Enhancement of anti-tumor activity of 1-beta-D-arabinofuranosylcytosine by encapsulation in liposomes. Int. J. Cancer, *20:* 581–587, 1977.
10. Fichtner, I., Reszka, R., Elbe, B., and Arndt, D. Therapeutic evaluation of liposome-encapsulated Daunoblastin in murine tumor models. Neoplasma (Bratisl.), *28:* 141–149, 1981.
11. Forssen, E. A. Chemotherapy with anthracycline-liposomes. *In:* G. Gregoriadis (ed.), Liposomes as Drug Carriers, pp. 355–364. New York: John Wiley & Sons, 1988.
12. Woo, S. Y., Dilliplane, P., Rahman, A., and Sinks, L. F. Liposomal methotrexate in the treatment of murine L1210 leukemia. Cancer Drug Delivery, *1:* 59–62, 1983.
13. Olson, F., Mayhew, E., Maslow, D., Rustum, Y., and Szoka, F. Characterization, toxicity and therapeutic efficacy of Adriamycin encapsulated in liposomes. Eur. J. Cancer Clin. Oncol., *18:* 167–176, 1982.
14. Rahman, A., White, G., More, N., and Schein, P. S. Pharmacological, toxicological and therapeutic evaluation in mice of doxorubicin entrapped liposomes. Cancer Res., *45:* 796–803, 1985.
15. Forssen, E. A., and Tokes, Z. A. Attenuation of dermal toxicity of doxorubicin by liposome encapsulation. Cancer Treat. Rep., *67:* 481–484, 1983.
16. van Hossel, Q. G. C. M., Steerenberg, P. A., Crommelin, D. J. A., van Dijk, A., van Oost, W., Klein, S., Douze, J. M. C., de Wildt, D. J., and Hillen, F. C. Reduced cardiotoxicity and nephrotoxicity with preservation of antitumor activity of doxorubicin entrapped in stable liposomes in the LOU/M Wsl Rat. Cancer Res., *44:* 3698–3705, 1984.
17. Kataoka, T., and Kobayashi, T. Enhancement of chemotherapeutic effect by entrapped 1-beta-D-arabinofuranosylcytosine in lipid vesicles and its mode of action. Ann. NY Acad. Sci., *308:* 387–394, 1978.
18. Mayhew, E., Papahadjopoulos, D., Rustum, Y. M., and Dave, D. Use of liposomes for the enhancement of the cytotoxic effects of cytosine arabinoside. Ann. NY Acad. Sci., *308:* 371–386, 1978.
19. Richardson, V. J., Jeyasingh, K., Jewkes, R. F., Ryman, B. E., and Tattersall, M. H. N. Possible tumour localization of Tc-99M-labelled liposomes: effects of lipid composition, charge and liposome size. J. Nucl. Med., *19:* 1049–1054, 1978.
20. Proffitt, R. T., Williams, L. E., Presant, C. A., Tin, G. W., Wiana, J. A., Gamble, R. C., and Baldeschweiter, J. D. Tumor imaging potential of liposomes loaded with In-III-NTA: biodistribution in mice. J. Nucl. Med., *24:* 45–51, 1983.
21. Gabizon, A., Peretz, T., Ben-Yosef, R., Catane, R., Biran, S., and Barenholz, Y. Phase I study with liposome-associated Adriamycin preliminary report. Proc. Am. Soc. Clin. Oncol., *5:* 43, 1986.
22. Treat, J., and Wholley, P. Liposome encapsulated doxorubicin: its role in cancer chemotherapy. J. Cell. Biochem. Suppl., *12B:* 257, 1988.
23. Kirby, C., and Gregoriadis, G. The effect of lipid composition of small unilamellar liposomes containing melphalan and vincristine on drug clearance after injection into mice. Biochem. Pharmacol., *32:* 609–615, 1983.
24. Layton, D., and Trouet, A. A comparison of the therapeutic effects of free and liposomally encapsulated vincristine in leukemia mice. Eur. J. Cancer, *16:* 949–950, 1980.
25. Mayer, L. D., Bally, M. B., Hope, M. J., and Cullis, P. R. Techniques for encapsulating bioactive agents into liposomes. Chem. Phys. Lipids, *40:* 333–346, 1986.
26. Mayer, L. D., Ko, D. S. C., Masin, D., Ginsberg, R. S., Cullis, P. R., and Bally, M. B. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res., *49:* 5922–5930, 1989.
27. Mayer, L. D., Wong, K. F., Menon, K., Chong, C., Harrigan, P. R., and Cullis, P. R. Influence of ion gradients on the transbilayer distribution of dibucaine in large unilamellar vesicles. Biochemistry, *27:* 2053–2060, 1988.
28. Bally, M. B., Mayer, L. D., Loughrey, H., Redelmeier, T., Madden, T. D., Wong, K., Harrigan, P. R., Hope, M. J., and Cullis, P. R. Dopamine accumulation in large unilamellar vesicles systems induced by transmembrane ion gradients. Chem. Phys. Lipids, *47:* 97–107, 1988.
29. Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim. Biophys. Acta, *817:* 193–196, 1986.
30. Mayer, L. D., Hope, M. J., and Cullis, P. R. Vesicles of various sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta, *816:* 294–302, 1985.
31. Williams, D. A. Interval estimation of median lethal doses. Biometrics, *42:* 641–645, 1986.
32. Bally, M. B., Hope, M. J., van Echteld, C. J. A., and Cullis, P. R. Uptake of safranine and other lipophilic cations into model membrane systems in response to a membrane potential. Biochim. Biophys. Acta, *812:* 66–76, 1985.
33. Mayer, L. D., Bally, M. B., Hope, M. J., and Cullis, P. R. Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential. Biochim. Biophys. Acta, *816:* 294–302, 1985.
34. Finkelstein, M. C., Kuden, S. H., Scheinen, H., Weissman, G., and Hoffstein, S. Liposome uptake by human leukocytes. Biochim. Biophys. Acta, *673:* 286–302, 1981.
35. Hsu, M. J., and Juliano, R. L. Interactions of liposomes with the reticuloendothelial system. II. Nonspecific and receptor-mediated uptake of liposomes by mouse peritoneal macrophages. Biochim. Biophys. Acta, *720:* 411–419, 1982.
36. Jackson, D. V., and Bender, R. A. Cytotoxic thresholds of vincristine in a murine and a tumor leukemia cell line *in vitro*. Cancer Res., *39:* 4346–4349, 1979.
37. Johnson, I. S., Armstrong, J. G., Gorman, M., and Bennett, J. P. The *Vinca* alkaloids: a new class of oncolytic agents. Cancer Res., *23:* 1390–1427, 1963.
38. Hwang, K. J. Liposome pharmacokinetics. *In:* M. Ostro (ed.) Liposomes from Biophysics to Therapeutics, pp. 109–156. New York: Marcel Dekker, Inc., 1987.
39. Jackson, D. V., Sethi, V. S., Spurr, C. L., Willard, V., White, D. R., Richards, F., Stuart, J. J., Muss, H. B., Cooper, M. R., Homesley, H. D., Jobson, V. W., and Castle, M. C. Intravenous vincristine infusion: phase I trial. Cancer (Phila.), *48:* 2559–2564, 1981.
40. Weber, W., Nagel, G. A., Nagel-Studer, E., and Albrecht, R. Vincristine infusion: a phase I study. Cancer Chemother. Pharmacol., *3:* 49–55, 1979.