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Antitumor and toxicity evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes*

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Summary. The antitumor activity of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes was evaluated in P388 ascitic leukemia, disseminated Gross leukemia, and advanced mammary carcinoma. In P388 leukemia, free drug and drug entrapped in liposomes demonstrated equivalent antitumor activity at doses of 2.2 and 4.4 mg/kg, demonstrating 52% and 69% ILS (increase in life-span), respectively. Free doxorubicin at a dose of 10 mg/kg was superior, producing a 185% ILS against 82% with liposomal doxorubicin. With an increase in administered dose the antitumor response with liposomal doxorubicin was much more pronounced; at doses of 20 and 25 mg/kg the ILS was in excess of 376%, with five of ten mice surviving tumor-free. In Gross leukemia, the optimum dose of free doxorubicin, 10 mg/kg, brought about 186% T/C (median survival in treated mice over that in controls, $\times 100$), whereas with liposomal doxorubicin the optimum dose was 16.9 mg/kg, which yielded 214% T/C. In advanced mammary carcinoma, the maximum tumor regression with free doxorubicin was at a dose of 7.5 mg/kg, with two of six mice dying of toxicity. Liposomal doxorubicin caused maximum tumor regression at 10.8 mg/kg dose with no toxic deaths. Doxorubicin entrapped in cardiolipin liposomes was much less toxic than free drug at high doses in normal mice.

Introduction

Anthracyclines such as doxorubicin and daunorubicin are active against a wide variety of human neoplasms. However, effective clinical use of these compounds has been hampered by serious dose-related cardiomyopathy [2, 12, 13]. Efforts have been directed at finding a means of reducing this toxicity while maintaining the therapeutic efficacy of these agents. Recently, Juliano and Stamp [11] have demonstrated that liposomes can serve as effective carriers of anthracyclines by altering the pharmacokinetics of these agents in rats. Subsequently, the use of liposomes as carriers of doxorubicin has been demonstrated to offer important advantages with regard to the attenuation of the dose-dependent cardiotoxicity. This effect has been shown in rodents [5, 7, 14, 17–19] and is apparently at least partly attributable to the reduced uptake of doxorubicin in cardiac tissue when it is administered entrapped in liposomes. Recently, our laboratories have demonstrated [10] that doxorubicin administered entrapped in cardiolipin liposomes over a long period affords complete protection from drug-induced cardiotoxicity in beagle dogs. These findings have generated considerable interest and suggest that this carrier system of doxorubicin may have clinical applicability. A critical step in the assessment of this possibility would be to demonstrate that antitumor properties of liposome-entrapped drug are fully expressed in various tumor models.

In the present study, we compared the therapeutic efficacy of doxorubicin entrapped in cardiolipin liposomes and of free doxorubicin against murine ascitic P388 leukemia, disseminated Gross leukemia, and advanced mammary carcinoma. A dose–response relationship was established in each tumor model. In addition, lethal toxicity of doxorubicin and doxorubicin entrapped in liposomes was evaluated in mice as a function of the dose of the drug. Our results strongly suggest that the lethal toxicity of doxorubicin is greatly reduced when it is entrapped in liposomes, which significantly enhances the therapeutic index of the drug in each tumor type studied.

Materials and methods

Murine ascites P388 leukemia was maintained by serial passage IP in DBA/2 female mice. For antitumor studies, 1×10^6 cells of P388 leukemia were implanted IP in CDF_1 male mice. At 24 h after tumor implantation, mice were treated IP with either free doxorubicin IP or doxorubicin entrapped in liposomes IP. Treatment was assessed by measuring survial time.

Gross leukemia was maintained by serial IV passage of an homogenate of spleen and peripheral lymph nodes of leukemic mice in C_3H/He syngeneic mice. Mice were treated IV on day 1 after tumor inoculum. Doxorubicin, either as free drug or entrapped in liposomes, was administered to mice via a lateral vein at 2% (0.02 ml/g_m) body weight. Antitumor efficacy was expressed in leukemic mice by the increase in median survival time over the dose range used, compared with survival in controls. Toxicity was established on the basis of the macroscopic necropsy findings recorded in all dead mice, as reported presviously [4].

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Antitumor activity against mammary carcinoma was evaluated in C_3H female mice inoculated SC in the mammary tissue with 2×10^7 cells from a third-generation tumor [4]. Treatment was performed either with free doxorubicin or with doxorubicin entrapped in cardiolipin liposomes, given IV via a lateral tail vein once a week for 4 weeks starting when tumors were palpable. Multiple IV injections of free doxorubicin produce extreme venous sclerosis, and the utmost care should be taken to avoid extravasation of the drug. However, with liposomal doxorubicin venous sclerosis following multiple injections of the drug appears to be substantially removed. Tumor growth was assessed by caliper measurement and tumor weight was calculated according to Geran et al. [8]. Non-tumor-bearing C_3H female mice were treated in parallel with the tumor-bearing mice and were observed until day 90.

For the comparison of lethal toxicity of free doxorubicin and doxorubicin entrapped in cardiolipin liposome, normal CDF_1 mice received single doses of 30.0, 22.1 and 16.4 mg/kg IV. The mice were observed until day 104 after drug administration. The control mice received either saline or blank liposomes representing the same concentration of the lipid as was used to entrap a dose of 30 mg/kg doxorubicin. Mice in each group were weighed twice weekly and were observed daily.

Preparation of liposomes

Doxorubicin was generously provided by Farmitalia Carlo Erba, Milan, Italy. Phosphatidyl choline, cardiolipin and cholesterol were obtained from Sigma Chemical Co. (St. Louis, Miss., USA), and stearylamine was obtained from K & K Laboratories (New York, NY, USA). The lipids were tested for purity by thin-layer chromatography on silica gel with a solvent system of chloroform/methanol/water 70:30:5 (vol./vol.) and were found to be pure. For antitumor evaluation of entrapped doxorubicin, liposomes were prepared by mixing 78.7 μmol drug with 39.3 μmol cardiolipin. The mixture was evaporated to dryness under nitrogen, and 200 μmol phosphatidyl choline, 136.8 μmol cholesterol, and 77.9 μmol stearylamine were then added. The mixture was stirred gently to achieve a homogeneous solution and then evaporated to dryness under N_2. The dried lipids were resuspended in 20 ml 0.01 M phosphate buffer with 0.85% NaCl. After a 30-min swelling time the liposomes were stirred for 15 min, followed by sonication (Heat System Model W-220F) in a fixed-temperature bath at 37 °C for 90 min. The nonentrapped doxorubicin was separated from liposome-encapsulated drug by extensive dialysis against 0.001 *M* phosphate buffer with 0.85% NaCl, pH 7.4 at 4 °C over a period of 20 h with at least two changes of buffer solution. The doxorubicin content of liposomes was determined from samples diluted in 50% ethanol – 0.3 *N* HCl [1], fluorometrically with an Aminco Bowman Spectrofluorometer with 490 nm excitation and 590 nm emission as described elsewhere [21]. Between 50 and 55% of the initial amount of doxorubicin was encapsulated in the liposomes and where necessary these were diluted with PBS so as to administer doses equivalent to those of free drug.

Results

Therapeutic efficacy of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes was evaluated against P388 leukemia in CDF_1 mice. Groups of ten mice received 1×10^6 cells by IP injection and treatment was initiated 1 day after tumor cell inoculation with either form of the drug. Table 1 shows the dose–response relationships of free doxorubicin and doxorubicin in liposomes against P388 leukemia. Free drug and drug entrapped in liposomes demonstrated equivalent antitumor activity at doses of 2.2 mg/kg and 4.4 mg/kg, with 52% and 69% increases in median life-span (ILS) for free doxorubicin and 50% and 68% ILS for doxorubicin liposomes, respectively. At a dose of 10 mg/kg free doxorubicin was superior, producing 185% ILS whereas with liposomal doxorubicin the ILS achieved was 82%. With an increase in the dose administered, the antitumor response with liposomal doxorubicin was much more pronounced; at doses of 20 and 25 mg/kg the ILS was in excess of 376%, with five of ten mice in each treatment groups surviving tumor-free for more than 50 days.

Figure 1 compares the efficacy of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes in the treatment of P388 leukemia at doses of 20 and 25 mg/kg. Mice treated with free drug showed median survival times of 8.5 and 9.5 days, respectively, and succumbed to toxicity-related deaths. Liposome-encapsulated doxorubicin led to toxic deaths in three of the ten mice at the 25-mg/kg dose, but those mice which escaped toxicity survived for more than 50 days. The same survival was achieved with

Table 1. Antitumor evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes against P388 leukemia

Dose mg/kg	Free doxorubicin			Doxorubicin-liposomes		
	Median survival time in days	ILS%	Long-term survivors	Median survival time in days	ILS%	Long-term survivors
2.2	17.5	52	0/10	16.5	50	1/10
4.4	19.5	69.5	0/10	18.5	68.0	0/10
6.6	16.5	43.5	1/10	21.5	95.0	1/10
10	31.5	186.0	6/20	20.5	82	2/20
15	22.5	105.0	3/20	26.5	136	4/20
20	8.5	−21.0	0/10	50.0	376	5/10
25	9.5	−10.0	0/10	50.0	376	5/10
7.5 qd 1, 3, 7	16.5	55	0/20	26	144	0/20

Mice were implanted IP 1×10^6 cells of murine P_{388} leukemia and 24 h later, free or liposome-entrapped doxorubicin was administered IP

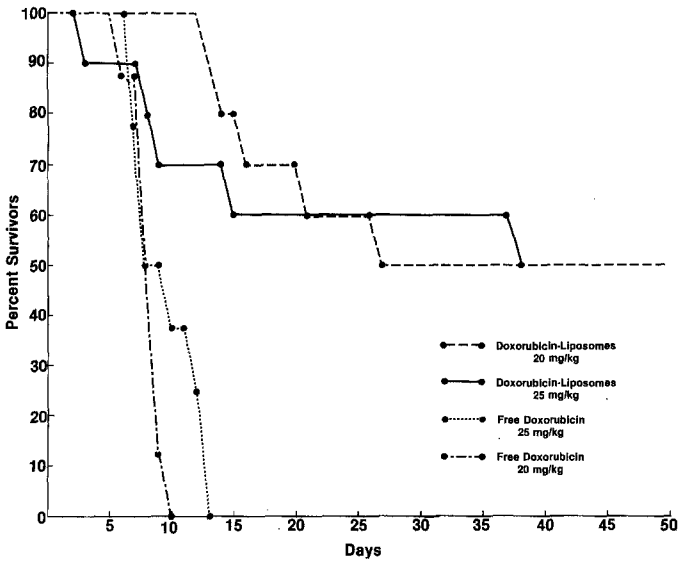

Fig. 1. Comparison of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes in the treatment of P388 ascitic leukemia. CDF_1 mice received IP implants at 1×10^6 cells of P388 leukemia and 24 h later drug doses of free doxorubicin and doxorubicin entrapped in liposomes were administered IP

mice receiving 20 mg/kg doxorubicin entrapped in cardiolipin liposomes. The surviving mice in both treatment groups were observed until day 104, and the experiment was terminated at that time.

Table 1 presents the effect of the schedule of drug administration on the response of P388 leukemia. In this experiment, tumor-bearing mice received IP free doxorubicin or doxorubicin entrapped in cardiolipin liposomes by IP injection on day 1 at a dose of 7.5 mg/kg, followed by additional doses on days 3 and 7. This regimen produced a 55% ILS with free doxorubicin, as against a 144% ILS with doxorubicin entrapped in cardiolipin liposomes. This schedule seems to be more effective in increasing the life-span of tumor-bearing mice with liposome-entrapped doxorubicin.

Disseminated Gross leukemia

Antitumor evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes was carried out in disseminated Gross leukemia in C_3H/He syngeneic mice. Drug treatment was started on day 1 with varying doses of free doxorubicin or doxorubicin in liposomes administered by IV injection, as shown in Table 2. The optimum dose of free doxorubicin was 10 mg/kg, which produced a T/C (median survival time of treated mice/median survival time of control mice $\times$ 100) of 186. With liposomal doxorubicin, the optimum dose (at which no deaths occured) was 16.9 mg/kg, which led to a T/C of 214%. Once again it was apparent that liposomal doxorubicin was less toxic than free doxorubicin, with tolerance of doses as high as 16.9 mg/kg, while this dosage of free doxorubicin was toxic in 100% of animals.

Advanced mammary carcinoma

For further evaluation of the effectiveness of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes, antitumor studies were performed against the advanced mammary carcinoma in C_3H female mice (Table 3). In all, four doses of either free drug or drug entrapped in liposomes were adminstered IV 1 week apart. Tumor weight was assessed at the end of the experiment. At a dose of 6 mg/kg free doxorubicin the tumor weight was 0.06 g (compared with 5.28 g in controls), demonstrating a 100% tumor growth inhibition. There were no toxic deaths at this dose in normal mice. The dose of 7.5 mg/kg free doxorubicin reduced tumor growth to 0.017 g (essentially full tumor regression) with two of six mice dying of toxicity. Liposomal doxorubicin at doses of 6 and 7.5 mg/kg was less effective in tumor inhibition than free drug, but at doses of 9 and 10.8 mg/kg it was equally effective. It is also evident that at the highest dose tested, 10.8 mg/kg, doxorubicin liposomes inhibited tumor growth to the same extent as free doxorubicin at the dose of 7.5 mg/kg, but did not induce any toxic deaths.

Comparison of lethal toxicity in normal mice with free doxorubicin and doxorubicin entrapped in liposomes

The antitumor studies in P388 leukemia, Gross leukemia, and advanced mammary carcinoma have indicated that liposomal doxorubicin is better tolerated at higher doses than is free drug. To determine the degree of protection from lethal toxicity, studies were carried out in normal CDF_1 mice with free doxorubicin and doxorubicin en-

Table 2. Antitumor evaluation of free and liposome-entrapped doxorubicin against disseminated gross leukemia

Compound	Dose (mg/kg)	T/c[a] (%)	No. of toxic deaths/total[b] no. of mice examined
Placebo		100	0/8
Doxorubicin	10	186	0/8
	13	200	2/8
	16.9	86	8/8
Doxorubicin liposomes	13	171	0/8
	16.9	214	0/8
	21.9	229	3/8
	30	86	8/8

[a] C_3H mice received 2×10^6 leukemia cells IV on day 0. Compounds were administered IV on day 1 after transplantation. A group of control mice was treated with blank liposomes

[b] Median survival time of treated mice/median survival time of control mice $\times$ 100. Toxicity was evaluated on the basis of macroscopic necropsy findings

Table 3. Antitumor evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes against advanced mammary carcinoma

Compound	Dose (mg/kg/week)	Mice with tumors			Normal mice
		Tumor[a] weight (g)	Tumor growth (+)[b] or regression (−) (%)	Tumor growth[c] inhibition (%)	Toxic deaths[d]
Control	–	5.28	+6698		0/6
Placebo	–	3.41	+7184		0/6
Doxorubicin	6	0.06	− 15	100	0/6
	7.5	0.017	− 79	100	2/6
Doxorubicin liposomes	6	0.67	+ 764	89	0/6
	7.5	0.134	+ 50	99	0/6
	9	0.06	− 27	100	0/6
	10.8	0.014	− 80	100	0/6

Drugs administered IV once a week for 4 weeks starting when tumors were palpable

[a] Evaluated 1 week after the end of treatment

[b] $\frac{\text{Tumor wt. at end of treatment}}{\text{Tumor wt. at beginning of treatment}} \times 100$

[c] Tumor growth inhibition evaluated for each group with respect to its own control

[d] Non-tumor bearing mice observed for at least 90 days after the start of treatment

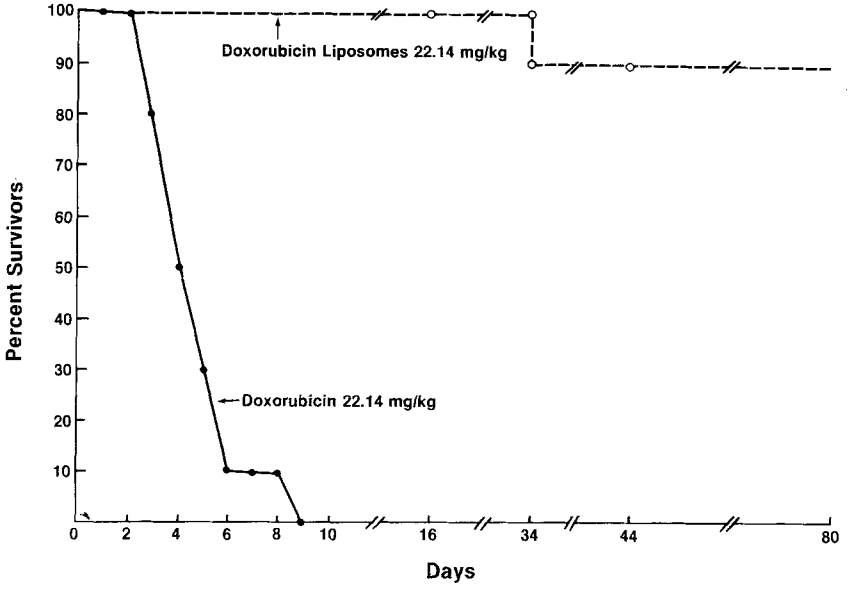

Fig. 2. Comparison of lethal toxicity of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes in normal CDF_1 mice. Groups of 10 mice received free doxorubicin 22.1 mg/kg or doxorubicin in liposomes, 22.1 mg/kg IV. Mice were observed each day and dead mice were autopsied for evaluation of toxicity

trapped in cardiolipin liposomes. Animals were randomly divided into groups of ten and received injections of free drug or drug entrapped in liposomes with single doses of 30.0, 22.1, and 16.4 mg/kg, respectively. Control mice received blank liposomes, the dose of lipids being 420 mg/kg. Animals treated with free doxorubicin at a dose of 30 mg/kg exhibited 100% mortality by day 4. However, animals treated with doxorubicin entrapped in cardiolipin liposomes at a dose of 30 mg/kg demonstrated only 10% mortality by day 4, with 90% of the animals surviving until day 30. With observation intervals in excess of 30 days greater mortality was observed in this treatment group, and 60% of the animals were dead by day 76.

Figure 2 compares the mortality rate of free drug and drug entrapped in liposomes at a dose of 22.1 mg/kg IV. With free drug 100% mortality was observed by day 9, whereas all the mice treated with liposomal doxorubicin survived until day 34. However, on day 36 one animal died in this dose group, and 90% of animals survived until day 90. A dose of 16.4 mg/kg free doxorubicin given IV demonstrated lethality in 50% of the mice by day 10, the remaining animals dying by day 56; no mortality was observed with the same dose of drug entrapped in liposomes with an observation period of 90 days (data not shown).

Variation in body weight was recorded in all groups of mice treated either with free drug or drug entrapped in cardiolipin liposomes. Figure 3 presents the weight changes in mice treated with saline, blank liposomes, free doxorubicin at 30 mg/kg, and doxorubicin entrapped in liposomes at the same dose. The mice treated with saline or blank liposomes had a progressive increase in body weight until day 90. The increase in body weight of mice treated

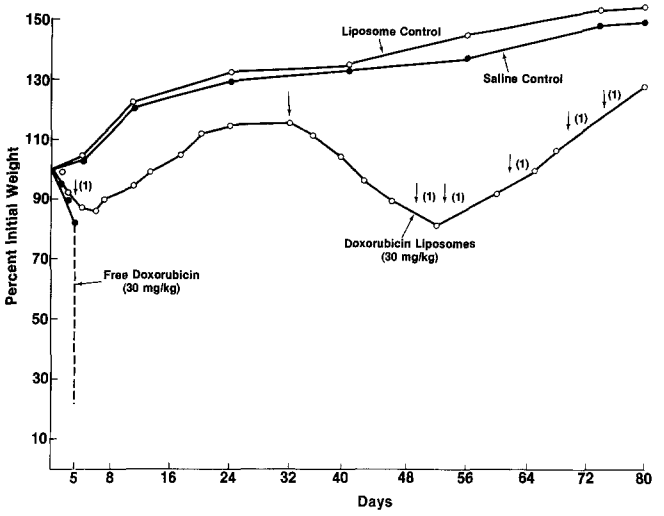

Fig. 3. Comparison of lethal toxicity of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes in normal CDF_1 mice. Mice (10 in each group) received free doxorubicin 30 mg/kg, doxorubicin entrapped in liposomes, 30 mg/kg, saline, or blank liposomes, by IV injection. The dose of lipids 420 mg/kg was equivalent to the amount used to entrap doxorubicin at a dose of 30 mg/kg. Mice were weighed each day for the first week and then twice a week for the duration of experiment, and the weight of each group of mice was calculated as a percentage of the initial weight. *Arrows*, deaths. *Figures in parentheses*, number of fatalities at each point

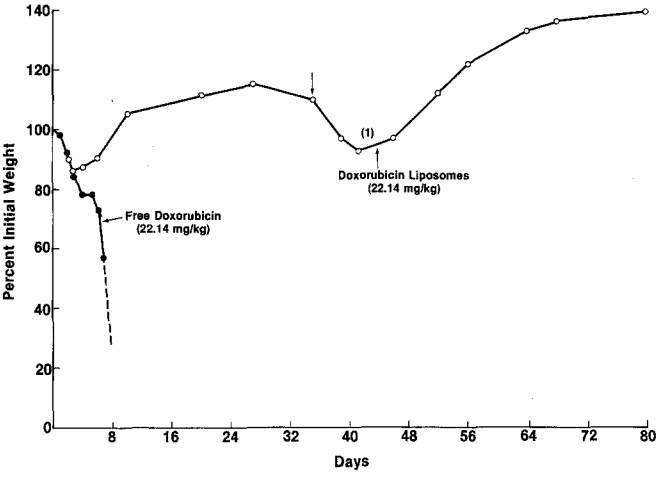

Fig. 4. Comparison of toxicity of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes in mice at a dose of 22.1 mg/kg. See legend to Fig. 3 for more detail

with blank liposomes was either equal to or greater than that recorded in mice treated with saline. Mice treated with free doxorubicin had a rapid weight loss, amounting to 15% by day 4, when all the mice were dead in this treatment group. Mice that received doxorubicin entrapped in liposomes at a dose of 30 mg/kg had a transient weight loss until day 8. A progesssive weight increase was observed in this group after day 9, which continued until day 32; at day 32 mice were observed to be extremely overactive and weight loss was observed in this group until day 52 with subsequent mortality.

Figure 4 presents the weight loss following free doxorubicin and doxorubicin entrapped in cardiolipin liposomes at a dose of 22.1 mg/kg. The mice treated with free doxorubicin lost over 40% of their initial body weight by day 7 and all the animals were dead by day 9. On the other hand, a very transient weight loss was observed in mice treated with liposomal drug.

In the mice surviving after a dose of 16.4 mg/kg free drug the same degree of weight loss was observed on day 34 as in mice treated with 30 mg/kg doxorubicin entrapped in liposomes. However, by day 56 all the animals treated with free doxorubicin at doses of 16.4 mg/kg were found dead (data not shown). In contrast, no significant variation in weight loss was seen in mice treated with liposome-entrapped doxorubicin at doses of 16.4 mg/kg (data not shown).

Discussion

The present study indicates that doxorubicin entrapped in cardiolipin liposomes can be safely administered at high doses and that this mode of administration is associated with a significantly increased life prolongation in various murine tumors. The antitumor activity is more or less comparable to that of free drug or drug entrapped in liposomes against P388 leukemia at lower doses (Table 1). Enhanced antitumor activity is observed when higher doses of drug are administered, however (Table 1). Antitumor studies in Gross leukemia with liposomal doxorubicin demonstrated a reduction in toxicity relative to the use of free drug, thereby providing a better therapeutic response at equiactive doses (Table 2). In advanced mammary carcinoma, which is considered a useful model for the study of human mammary cancer, equivalent therapeutic responses were seen with free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. At an optimal dose of 7.5 mg/kg, free doxorubicin effected 100% tumor regression, which was associated with 30% mortality of the mice. However, the optimum dose of doxorubicin entrapped in cardiolipin liposomes brought about the same degree of tumor regression but without any lethality (Table 3). The significant reduction in lethal toxicity in normal mice when doxorubicin was administered entrapped in cardiolipin liposomes rather than as free drug may be related to the altered pharmacodynamics [21] or an altered subcellular distribution of the drug [6].

Doxorubicin has been incorportated in liposomes of various compositions and prepared by different mehtods [5, 7, 15, 20] to alter the tissue distribution and reduce the cardiac toxicity. The present studies demonstrate that the intrinsic properties of liposome components can be exploited to cause an effective reduction in the toxicity of anthracycline drugs, thereby increasing the therapeutic ratio. In this respect Goormaghtigh et al. [9] have demonstrated a high affinity of cardiolipin for doxorubicin in a membrane model system. This affinity is thought to be stabilized by two essential interactions: an electrostatic interaction between the protonated amino groups of the sugar residues and the ionized phosphate residues, and an interaction between adjacent anthraquinone chromophores. We have utilized these properties in the preparation of stable doxorubicin liposomes, in contrast to the work reported by Gabizon et al. [7]. It appears that both a positive charge and the presence of cardiolipin are needed to improve the stability of these liposomes [22]. In fact, doxorubicin entrapped in cardiolipin liposomes has been demon-

strated in our laboratories to completely prevent drug-induced cardiotoxicity in mice [22] and in dogs [10]. Beagle dogs (5 in each group) received seven doses of 1.75 mg/kg free doxorubicin or doxorubicin entrapped in cardiolipin liposomes IV at 3-week intervals. Free doxorubicin administered according to this regimen caused extensive myocardial alterations in beagles, the prominent features being cytoplasmic vacuolization and loss of myofibrils. In contrast, the hearts of all dogs given liposomal doxorubicin at equivalent doses remained essentially normal, with cardiac morphology similar to that of saline-treated control animals despite administration of a cumulative dose of 245 mg/m^2.

The potential clinical benefit of doxorubicin entrapped in liposomes must be evaluated in the light of the reduced acute and chronic cardiotoxicity reported with this modality of administration [5, 14, 10, 17, 19]. The present studies indicate that general toxicities of doxorubicin entrapped in cardiolipin liposomes are significantly reduced, which makes higher doses of the drug possible. The enhanced therapeutic response accompanied by higher doses of drug in three murine tumor types provides the basis for the design of future clinical trials with this novel carrier system. Recently, liposomes have been administered in humans [3, 13], and they have been shown to be devoid of any toxicities in man. It is argued that anatomical barriers will determine differential access and differential antitumor activities of liposome-entrapped drugs, raising objections about the applicability of these carriers in cancer chemotherapy [17]. The present studies demonstrate that with the increase in dosage possible with entrapped drug a marked therapeutic benefit is achieved. This extends to the treatment of solid tumors, as demonstrated by the present studies in established mammary carcinoma. Hence, the liposomal carrier system for doxorubizin could be successfully exploited clinically, to control cardiotoxicity while allowing full expression of the therapeutic efficacy of this agent.

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