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Use of anionic liposomes for the reduction of chronic doxorubicin-induced cardiotoxicity

(adriamycin/phospholipid vesicles/antileukemic activity/phosphatidylcholine)

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ABSTRACT **Anionic liposomes containing doxorubicin were evaluated in mice for therapeutic potential in reducing the risks of chronic cardiotoxicity characteristic of long-term high-dose anthracycline therapy. Doxorubicin first was complexed to phosphatidylcholine and then entrapped in anionic vesicles. Quantitation of myocardial injury was accomplished through examination of thin sections of cardiac tissue by light microscopy. At treatment levels of either 20 or 40 mg/kg (total dose), mice receiving liposomal doxorubicin had toxicity scores indistinguishable from or only slightly greater than those of saline-treated controls. Similar total doses of free drug produced moderate to severe myocardial damage and yielded much higher toxicity scores. Mixture of free doxorubicin with empty liposomes did not alleviate cardiac toxicity, indicating that the drug must be entrapped within phospholipid vesicles for reduction in toxicity. The inhibition of body growth produced by free doxorubicin at both dose levels was also completely eliminated by encapsulation in liposomes. Doxorubicin liposomes were also tested for chemotherapeutic potential against L-1210 and P-388 murine leukemias. In all cases, treatment with liposomal doxorubicin produced increases in life-span greater than that observed for free drug. We conclude that anionic liposomes can function as efficacious carriers of doxorubicin. These vesicles possess improved therapeutic action as reflected by their ability to reduce cardiac toxicity, overcome growth inhibition, and increase antileukemic activity.**

The anthracycline antibiotic doxorubicin (Dxn) is an important antitumor agent with marked activity against a wide variety of human neoplasms (1). Chronic cardiotoxicity, however, has limited the clinical use of this drug in man (2). Current recommendations (3) suggest a safe total dose of about 550 mg/m^2 (corresponding to approximately 14 mg/kg of body weight for a male adult) although toxicities and death (4) have occurred at lower doses. Children are also highly susceptable to Dxn-induced congestive heart failure at lower doses (5). Long-term administration of doses exceeding the recommended amount lead to increased patient risk of insidious development of a distinct form of cardiomyopathy (6). In man, as well as in several animal models, this toxicity is characterized by sarcoplasmic vacuolar degeneration, loss of myofilaments, and progressive atrophy of myofibrils (7, 8). Ultrastructural changes include swelling of mitochondria with apparent breakup of crystae (9, 10).

Liposomes have received considerable attention as drug carriers for their ability to increase antitumor activity (11), alter *in vivo* tissue distribution (12) and decrease toxicity (13). Variations in endocytotic ability play an important role influencing the relative uptake of liposomes by different tissues (14, 15). The cells of the reticulo-endothelial system and highly endocytotic tumor cells display increased accumulations of liposomes and their contents. In contrast, cardiac myocytes have relatively little capacity for endocytosis and would thus be expected to take up only small amounts of liposome-entrapped material. Up until now, the difficulty in producing a Dxn-containing liposome was due to the detergent-like ability of Dxn to break up phospholipid vesicles. We recently described (16) the preparation of stable Dxn-liposomes containing a phosphatidylcholine (PtdCho)-Dxn complex which minimized the disruptive effects that this drug exerted on the bilayer. Here we report a significant decrease in chronic Dxn-induced cardiotoxicity and an increase in its antitumor activity brought about by entrapment in anionic liposomes.

MATERIALS AND METHODS

Preparation of Liposomes. Doxorubicin was kindly supplied by Adria Laboratories (Columbus, OH), PtdCho phosphatidylserine (PtdSer), and cholesterol (Chol) were purchased from Sigma. All compounds were tested for purity by thin-layer chromatography (TLC) on silica gel with chloroform/methanol, 90:10 (vol/vol), and chloroform/methanol/water, 65:25:4/(vol/vol), used for development. The lipids were found to be pure or, in a few samples of PtdCho to contain trace amounts of Chol. Dxn was found to be pure on silica gel TLC under conditions previously used for the separation of Dxn from its metabolites and breakdown products such as adriamycinol, adriamycinone, and aglycone (17). Dxn was complexed with PtdCho by adding a solution of the drug in 0.077 M NaCl to the dried lipid in a 1:2 molar ratio (Dxn/PtdCho). This mixture was sonicated at 35°C for 5 min per ml under nitrogen atmosphere by using a needle probe type sonicator (Braun Sonic, 1410) set at 100 W. The resulting complex was then entrapped within anionic liposomes consisting of PtdCho, PtdSer, and Chol in a molar ratio of 0.6:0.2:0.3 per mol of Dxn. This liposome suspension was sonicated as described above, again at 100 W for 5 min per ml. Liposomes were separated from unentrapped Dxn by gel filtration on a Sephadex G-50 column eluted with 0.154 M NaCl. After this procedure, 5–10% of the starting Dxn could be entrapped. The average diameter of these vesicles was about 800–1000 Å as determined by electron microscopy.

Organ Distribution. [^{3}H]Dxn was produced by custom labeling at Moravek Biochemicals (City of Industry, CA; specific activity, 600 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels). After incubation at pH 6.4 and 8.4 this material was found to be pure by the TLC methods described above. Swiss mice (Simonson Laboratories, Gilroy, CA), weighing about 20 g each, received [^{3}H]Dxn in either the free or entrapped form by tail vein injection. Mice were sacrificed at 1 and 4 hr after drug administration. Brain, heart, liver, lungs, and spleen were removed and prepared for liquid scintillation counting in a Beckman LS-

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Abbreviations: Dxn, doxorubicin, PtdCho, phosphatidylcholine, PtdSer, phosphatidylserine; Chol, cholesterol; TLC, thin-layer chromatography.

9000 instrument with Protosol as a tissue solubilizer. The distribution of [^{3}H]Dxn was expressed as a percentage of the total radioactivity measured for all organs examined.

High-Dose Cardiac Toxicity Study. Swiss mice weighing approximately 20 g each were given weekly tail vein injections of Dxn at 5 mg/kg. The groups studied were: drug entrapped in anionic liposomes, free drug plus empty liposomes (same Dxn/lipid ratio), free drug alone, and saline. All liposome and Dxn solutions were made up fresh for each injection. Mice were treated either four or eight times and received total doses of 20 or 40 mg/kg. During the 8-week trial, animals were not treated for a 2-week interval between the fourth or fifth injections, to allow for recovery of bone marrow depression. Mice were sacrificed by cervical dislocation either 12 (low-dose) or 13 weeks (high-dose) after the initial injection.

The hearts were divided into atrial and ventricular portions, and the latter were fixed in 3% paraformaldehyde/2% glutaraldehyde/1.6% cacodylate, pH 7.3. Tissue was then embedded in either Vestopol or glycol methacrylate and sections 1.0–1.5 μm were cut with a Sorvall JB-4A microtome. After staining with 0.1% toluidine blue, sections were observed by light microscopy under oil immersion at ×400 and ×1000. Two sections were prepared from each heart and mounted on separate slides. All slides were coded at random to permit unbiased observation and scoring. Evaluation was performed as a blind study by two independent reviewers who used the criteria for cardiac histotoxicity developed by Bertazzoli *et al.* (18). Scores for cardiac lesions were based on severity and extension. Two possible values could be assigned to severity: degree 1, sarcoplasmic microvacuolizations or cellular edema; degree 2, all the criteria for degree 1 plus sarcoplasmic macrovacuolization and cell necrosis. Extension values ranged from 0 to 5: 0, no visible lesions were observed; 5, ≥50% of the observable cells were damaged. The toxicity score was computed as the product of the severity and extension values.

***In Vivo* Antitumor Activity.** Stock ascitic L-1210 or P-388 tumor cells were supplied by T. Khwaja (Animal Tumor Resource Facility, University of Southern California Comprehensive Cancer Center). Tumor cells (10^5 suspended in 0.25 ml of RPMI-1640 tissue culture medium) were injected intraperitoneally into female DBA/2 mice (Simonson Laboratories, Gilroy, CA). Treatment commenced 1 day after tumor cell inoculation. Treated animals received three 5-mg/kg doses of Dxn in the entrapped form, as free drug plus empty liposomes, or as free drug alone. All liposome and Dxn solutions were made up fresh for each injection. Controls received isotonic saline only. L-1210 and P-388 tumor-bearing mice were treated at 5- and 7-day intervals, respectively. Estimation of antileukemic effectiveness for free and liposome-encapsulated drug was based on increases in mean survival time compared to controls. Long-term survivors were defined as those animals that lived longer than 30 days after inoculation. Statistical analyses were performed by using the one-tailed Student *t* test.

RESULTS

The relative cardiac uptake of Dxn injected as either the free or liposome encapsulated form was determined at 1 and 4 hr after administration. With encapsulated Dxn, cardiac tissue had mean (± SEM) relative accumulations of only 2.5 ± 0.7 and 1.4 ± 0.8% at 1 and 4 hr, respectively. Injection of the free drug produced substantially greater relative accumulations: 4.4 ± 0.5 and 3.5 ± 0.5% for the same periods.

High doses of free Dxn (20–40 mg/kg) inhibited the growth of immature mice (5–6 weeks old) (Table 1). Equal doses of free Dxn plus empty liposomes, containing a lipid content equal to that of Dxn-liposomes, also inhibited growth. This inhibition was not significantly different from that of the free drug ($P > 0.2$). When equal doses of Dxn were administered in the liposome-entrapped form, no suppression in weight gain could be detected.

Representative samples of cardiac tissue were observed with electron microscopy to confirm that the ultrastructural changes characteristic of Dxn toxicity had been induced. These lesions included sarcoplasmic vacuolization, fragmented sarcomers with disruption of the usual parallel arrangement of myofibers, and swollen mitochondria with disrupted cristae. A scattergram for the values obtained from experiments performed at two different dose levels is shown in Fig. 1.

Mice given a total dose of 20 mg/kg were evaluated in the first experiment. Based on 1.0-μm sections of cardiac tissue embedded in Vestopol, the group receiving Dxn in the free form had a mean (±SEM) toxicity index of 2.65 ± 0.81. In this group, widespread areas of myofibril degeneration, sarcoplasmic vacuolization, and myocardial necrosis were observed in those samples that were severly affected. In contrast, the group treated with Dxn-liposomes had a score of 0.99 ± 0.25 which was indistinguishable from that of the saline controls; none of the whole heart sections in this treatment group displayed any sign of severe tissue damage. Some areas of possible cellular edema were scored in the liposome group but these were of a lmited nature and occurred with the same frequency as in the saline control group which had a toxicity score of 1.33 ± 0.34. Any protective effect afforded by the mere combination of empty liposomes with free Dxn was ruled out because a toxicity score of 4.14 ± 1.06 was observed. The difference between free and liposome-mediated drug delivery was statistically significant, $0.05 < P < 0.1$.

A second study was conducted with a regimen yielding a total Dxn dose of 40 mg/kg. At this level, a greater proportion of animals receiving unmodified Dxn would be expected to develop cardiotoxicity. Evaluation of heart tissue embedded in glycol methacrylate and cut at 1.5-μm revealed that mice re-

Table 1. Effect of mode of delivery on Dxn suppression of body growth

	Low-dose			High-dose		
	Weight, g		Δ weight,*	Weight, g		Δ weight,*
Treatment	Start	At 12 weeks	%	Start	At 13 weeks	%
Saline control	19.4	29.9	54.3 ± 9.9	21.5	32.5	51.2 ± 6.4
Free Dxn	19.9	23.8	19.4 ± 13.4	21.8	25.5	16.7 ± 9.1
Free Dxn plus empty liposomes	19.2	26.6	38.5 ± 5.7	21.3	30.5	43.2 ± 6.7
Dxn-liposomes	18.7	29.0	55.3 ± 10.6	21.6	33.1	53.2 ± 7.3

Low-dose group (four animals per treatment) received a total of 20 mg/kg; high-dose group (six animals per treatment) received 40 mg/kg.

* Values are mean ± SEM. For difference between Dxn-liposome and free Dxn groups, $P < 0.05$.

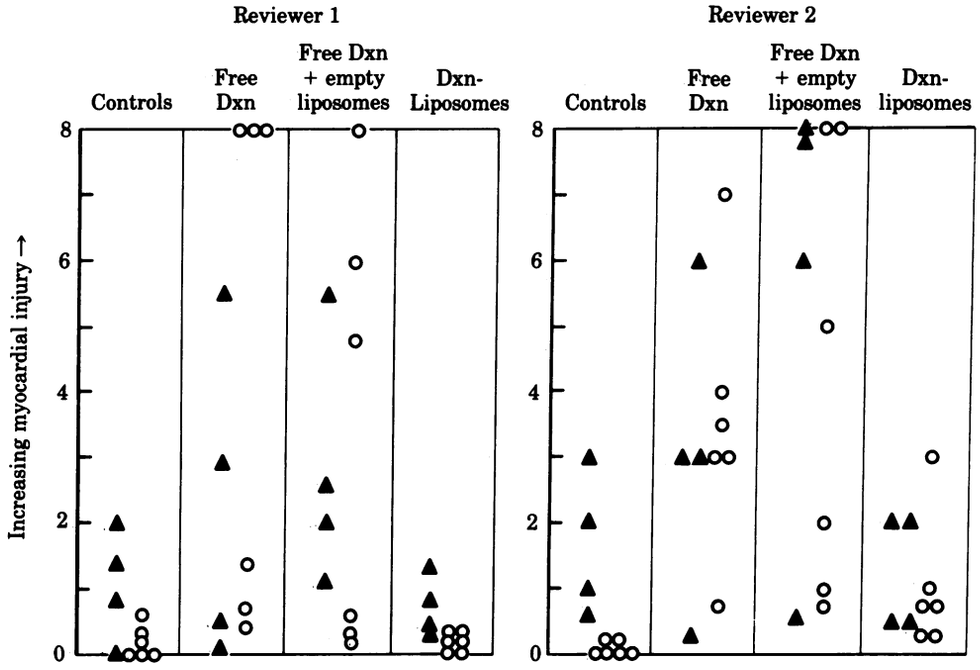

FIG. 1. Two representative slides of heart tissue from each test animal were scored by two reviewers who did not know the treatment received by the mice. Points shown are the average score for each animal heart reviewed. ▲, 20 mg/kg, total dose; ○, 40 mg/kg, total dose.

ceiving Dxn in the free form had a high toxicity score, 3.98 ± 0.87. Those animals receiving Dxn liposomes exhibited a markedly diminished toxicity score, 0.69 ± 0.23. This value was slightly greater than that of the saline control group score of 1.05 ± 0.5. This small difference raises the possibility that at this high dose of Dxn, liposome encapsulation did not completely eliminate all detectable toxicity. The apparent lesions in the Dxn-liposome group were noted by only one of the reviewers. In these cases, myocardial damage consisted of scarce sarcoplasmic microvacuolizations or dilated sarcoplasmic reticulum in a few single, isolated cells per cross section. No groups of affected cells or necrosis were identified in any of the slides from this treatment group. The toxicity score of 3.73 ± 0.94 for free drug plus empty liposomes again confirmed that Dxn must be entrapped within liposomes and not merely associated with them in order to reduce the toxicity. For this second study the assertion that a decrease in toxicity had been achieved by liposome encapsulation was highly significant ($P < 0.002$). The apparent discrepancy for the saline control values between the two different dose experiments is most likely due to variations in fixing and embedding techniques.

The *in vivo* antileukemic activity of Dxn-liposomes was compared to that of the free drug for the L-1210 and P-388 mouse leukemia systems (Table 2). In each experiment Dxn-liposomes were more effective than free drug for increasing life span ($P < 0.05$). The increase for all groups treated with free drug plus empty liposomes was comparable to that for those treated with free drug alone, further demonstrating that the observed therapeutic improvement was due to entrapment.

DISCUSSION

In order to provide a stable vesicle, a complex of Dxn and PtdCho was prepared and subsequently entrapped in phospholipid vesicles. Such double packing resulted in a vesicle that was stable even in the presence of serum at 37°C (16). The drug-phospholipid complex counteracts the destabilizing effect that Dxn exerts on phospholipid membranes (19) by increasing its partitioning into the aqueous phase (20) and minimizing its interaction with the bilayer. It is likely that this interaction is not completely eliminated, however, since the entrapment efficiency of 5–10% achieved with this procedure is greater than expected for a compound taken up only by the liposome aqueous phase.

Previous investigators have indicated that liposomes can function as protective vehicles by altering the normal patterns of drug distribution and decreasing the uptake of potentially toxic substances into sensitive tissues (13). Because of the large amount of drug required for the toxicity experiments, it was decided to focus initially on one liposome type. The rationale for this study considered two molecular aspects for the design of liposomes. First, Chol was added to liposomes in order to increase their rigidity and to increase their cellular uptake by the phagocytic route (21). This was considered beneficial because a relatively low level of phagocytic activity is seen in cardiac myocytes compared to cells of the reticuloendothelial system. This should result in a decreased myocardial uptake. The second aspect is related to the net negative charge of the liposomes used in this study brought about by the inclusion of PtdSer in the bilayer. Because the heart is one of the most highly perfused organs in the body, it is exposed to the highest levels of circulating drug after an intravenous injection. The decreased association of Dxn with cardiac tissue produced by liposome entrapment may be due in part to the net negative charge of the bilayer. Such a net negative charge may result in a decreased interaction with the negative surfaces of endothelial cells, thereby decreasing uptake by surface adsorption or fusion in cardiac tissue.

The acute toxicity of Dxn has been associated with single doses (40 mg/m^2) which can bring about cardiac arrhythmias and in some cases even death (4). During normal clinical use these abnormalities are usually transient and many reverse spontaneously during or after therapy. In a recent report, Rahman *et al.* (22) concluded that acute cardiac damage could be

Table 2. Antileukemic activity of anionic DXN-liposomes and free drug

Leukemia cell type*	Mode of Dxn treatment	n	Long-term survivors, no.	Increase, in life-span,%
	Trial 1			
L-1210	Dxn-liposomes	6	3	123
	Free Dxn + empty liposomes	5	0	16
	Free Dxn	4	1	18
	Trial 2			
L-1210	Dxn-liposomes	6	0	16
	Free Dxn + empty liposomes	6	0	7
	Free Dxn	6	0	3
	Trial 3			
P-388	Dxn-liposomes	7	1	58
	Free Dxn + empty liposomes	7	0	19
	Free Dxn	6	2	17
	Trial 4			
P-388	Dxn-liposomes	6	2	93
	Free Dxn + empty liposomes	6	0	68
	Free Dxn	6	0	63

Results of four experiments comparing antitumor activites of Dxn-liposomes and of free drug against L-1210 and P-388 murine leukemias. DBA/2 mice received 10^5 leukemia cells by the intraperitoneal route. This was followed by three intraperitoneal injections of Dxn at 5 mg/kg at weekly intervals (total dose, 15 mg/kg), commencing 1 day after tumor cell inoculation. Long-term survivors are defined as those animals living 30 days or more after tumor cell inoculation. The percentage increase in life-span is calculated relative to saline controls and is based on those animals not living to become long-term survivors.
* Cells for trial 1 were grown in cell culture and then passed twice through DBA/2 mice. Cells for trials 2–4 were direct passages from DBA/2 mice.

reduced by administering Dxn entrapped in positively charged liposomes. Chronic cardiac toxicity, however, is a more life-threatening situation and is most often unresponsive to supportive therapy. (2). It correlates more closely with the total dose but can be minimized by administering low doses over a prolonged period. When evaluating the chronic toxicity with animal models it is therefore important to approximate the clinical use of the drug by giving doses spaced at weekly intervals rather than using a large bolus or daily injections (18). In patients as well as in animal models, the chronic toxicity may require months of progression prior to its clinical manifestation. Histological changes occur earlier, requiring only a few weeks to become detectable by light microscopy (23). Previous investigators have recommended light microscopy for quantitative experimental evaluation of Dxn cardiotoxicity because it allows observation of larger amounts of tissue than is practical with electron microscopy.

Because test animals can display vastly different sensitivities toward the total Dxn dose these toxicity studies used the relatively high doses of 20 and 40 mg/kg. This ensured that a large percentage of animals developed cardiotoxicity. Quantitation of Dxn-induced myocardial lesions revealed that severe tissue damage could be produced at both levels of free drug used in this study. At the lower dose, 20 mg/kg, liposomal delivery of Dxn eliminated all observable toxicity. The higher dose, 40 mg/kg, produced only a low incidence of lesions when Dxn-liposomes were given. The toxicity score was substantially lower than that seen for the free drug and approached that of saline controls. Additional support for the protective potential of liposome encapsulation is found in the observation that Dxn-liposome-treated animals did not display the same inhibition of body growth that had been produced by treatment with the free drug. The data thus indicate that these anionic, double-packed Dxn-liposomes are able to reduce both cardiac toxicity and overall systemic toxicity as evaluated by normal body growth.

Using the L-1210 or P-388 murine leukemia models recommended for the primary screening of anthracyclines (24), the current study has demonstrated that Dxn entrapped in anionic liposomes is more effective than free drug for increasing life-span. Thus, these liposomes prepared by the double-packing technique not only protect against anthracycline-induced cardiomyopathies and systemic toxicity but also provide greater anti-tumor activity. This contrasts with the earlier work on positively charged liposomes used in acute cardiac damage studies which found no improvement in antileukemic activity (22). Although the mechanism by which the increase in life-span is brought about remains unknown, liposome encapsulation may increase Dxn availability to leukemic cells by decreasing nonspecific tissue binding and giving preference to phagocytic uptake. When using ascitic tumor models, one should consider a possible bias which would favor increased liposome activity over free drug activity as a result of intraperitoneal injection. Using labeled phospholipid, previous investigators have found, that, at 1 hr after injection, the tissue distribution of anionic small unilamellar vesicles given intraperitoneally was qualitatively although not quantitatively, similar to that produced by intravenous administration (25). For the tissues, investigated the total uptake after intraperitoneal administration was about 70% of that for the intravenous route. In anti-tumor studies, cytosine arabinoside encapsulated in anionic multilamellar vesicles and administered intraperitoneally for ascitic L-1210 produced increases of life-span similar to those found by using the intravenous route of treatment for disseminated L-1210 (increases of 229% and 183%, respectively. For both tumor models, the liposomes proved superior to free drug which produced increases of life-span of 119% for intraperitoneal and 103% for intravenous administration. The disseminated tumor, however, did require an intravenous dose of 10 mg/kg for both free drug and anionic multilamellar vesicles. This was greater than the intraperitoneal dose of 4.5 mg/kg for free drug and 4.4 mg/kg for the vesicles anionic for treating the ascitic tumor.

Prior to acceptance as clinically useful methods of drug delivery, drug-liposome complexes must be shown to possess an increased therapeutic index. A recent study by Kaye *et al.* (26) demonstrated that, although liposome encapsulation of methotrexate could reduce the dose needed to inhibit tumor growth, it also reduced, by the same amount, the dose producing host toxicity. Thus, liposomes provided no improvement in the therapeutic index. The same investigators also studied cationic actinomycin D-liposomes which had previously been shown effective for overcoming drug resistance *in vitro*. However, when tested *in vivo* against actinomycin D-resistant ROS tumor, maximal nontoxic doses were still ineffective against the tumor. In this situation, liposomes again offered no therapeutic advantage.

The significance of our study lies in its clinical relevance. We have demonstrated that encapsulation nearly eliminates the characteristic cardiotoxicity at doses equal to or greater than those currently used in either pediatric or adult oncology. This is accompanied by elimination of all significant Dxn-induced suppression of normal growth seen in young mice. This obser-

vation indicates that children who must be placed on anthracycline chemotherapy may experience a reduction in toxicity when receiving Dxn in the liposome form. This decrease in host toxicity is associated with a significant increase in antileukemic activity, establishing a substantial improvement in the therapeutic index of Dxn.

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