Eur J Cancer Clin Oncol, Vol. 18, No. 2, pp. 167–176, 1982.
Printed in Great Britain.

0277-5379/82/020167-09$03.00/0

Characterization, Toxicity and Therapeutic Efficacy of Adriamycin Encapsulated in Liposomes*

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Abstract—*The inherent toxicities of drug-free liposomes were compared. Positively charged liposomes were more toxic* in vitro (*chick heart cells*) *and* in vivo (*5-fold reduction in* LD_{50} *in mice*) *than neutral or negatively charged liposomes. Based on these findings, adriamycin was encapsulated in negatively charged liposomes* (*PG*: *PC*: *Chol*—1:4:5), *sequentially extruded through polycarbonate membranes* (*to* 0.2 μm *pore size*) *and purified by exhaustive dialysis. This preparation had a mean vesicle diameter of* 0.24 μm *and was stable in serum* (24 hr *drug retention of* 85%). *As compared with free drug, liposome-encapsulated adriamycin was less toxic* in vitro *to chick heart cells. In mice, adriamycin displayed short-* (4–14 day) *and long-* (45–70 day) *term toxicity. Encapsulating adriamycin increased both short-* (20–50 mg/kg) *and long-* (10–15 mg/kg *to* 25–30 mg/kg) *term* LD_{50} *levels. As compared with free drug, administering encapsulated adriamycin* in vivo *reduced the incidence of cardiac histopathologic lesions at* 20 *and* 40 mg/kg. *Compared* in vivo, *plasma levels of liposome-encapsulated adriamycin were* 2/3-*fold higher than free drug up to* 24 hr *post drug administration. Cardiac uptake of adriamycin was reduced* 2-*fold* (*conc.* × *time value for* 48 hr) *following encapsulated drug administration. Encapsulating adriamycin in liposomes did not alter its therapeutic effect against L*1210 *leukemic cells* in vivo.

INTRODUCTION

ADRIAMYCIN, an anthracycline antibiotic, is a potent chemotherapeutic agent effective against a broad spectrum of neoplasms [1]. Its clinical usefulness, however, is limited by its cardiotoxicity [2]; cardiac damage is cumulative and irreversible, with doses greater than approximately 550 mg/m^2 in humans associated with the development of congestive heart failure [2, 3].

One approach to reducing the cardiotoxicity of adriamycin is to alter the *in vivo* distribution pattern of the drug by using a delivery system such as liposomes or by linking adriamycin to other macromolecular carriers such as DNA [4, 5]. Such an approach has been shown to alter the tissue distribution patterns of liposome-encapsulated methotrexate [6], cytosine arabinoside [7] and actinomycin D [8]. Recently, altered cardiac uptake of encapsulated adriamycin has been demonstrated with both anionic [9] and cationic [10] liposomes.

The present study was designed to optimize the therapeutic properties of liposome-encapsulated adriamycin. Specifically, we investigated the relative toxicities of anionic, cationic and neutral liposomes to cultured chick heart cells *in vitro* and to mice *in vivo* and characterized the size distribution and serum stability of liposomes containing adriamycin. Defined liposome preparations containing adriamycin were then compared with free drug as to tissue distribution, toxicity and therapeutic effectiveness against L1210 leukemia in the mouse.

Accepted 3 September 1981.
*Supported by a grant from the American Heart Association (E.M.), and N.I.H. grants CA 28494 (E.M.), CA 14370 (D.M.), CA 13038 (Y.R.) and contract CM 77118 (E.M.) from N.I.H.
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Abbreviations: SA, stearylamine; PC, egg lecithin (phosphatidylcholine); PG, phosphatidylglycerol; Chol, cholesterol; MCCR, myocardial cell contraction rate; MLV, multilamellar vesicles; FCS, fetal calf serum; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetracetic acid; DNA, deoxyribonucleic acid.

MATERIALS AND METHODS

Preparation of liposomes

Adriamycin (NSC lot No. 123127-MT), dissolved in acetone at 0.5 μmol/ml, was mixed with egg phosphatidylglycerol (PG), egg phosphatidyl choline (PC) and cholesterol (Chol) in a 1:4:5 molar ratio dissolved in chloroform at an adriamycin: total lipid molar ratio of 1:20. Preparation of purified lipids and storage conditions have been previously described [11, 12]. The lipid mixture containing adriamycin was dried onto a 500 ml round-bottom flask with a rotatory evaporator. Phosphate-buffered saline (PBS) with 1 mM EDTA was added to the flask at 1 ml per 60 μM lipid and shaken at 37°C for 5 hr on an oscillatory shaker (S-500, Kraft Apparatus, Mineola, NY) at speed setting 5. Vesicles so formed were either used at this stage or further processed by sequential extrusion through 0.4 μ and 0.2 μ pore size polycarbonate membranes[13]. Vesicles of the same composition that did not contain adriamycin were also produced by this procedure for size comparison.

In addition, multilamellar liposomes bearing cationic (stearylamine (SA):PC:Chol, 1:4:5), neutral (PC:Chol, 1:4) and negative (PG:PC:Chol, 1:4:5) charges were prepared according to standard methodology [11].

Preparation of liposome-encapsulated adriamycin for in vivo *studies*

Vesicles, prepared as described above by extrusion through 0.2 μ pore size polycarbonate membranes, were dialyzed at 37°C for 30 hr using 4 changes of PBS–1 mM EDTA (1000 ml PBS–EDTA per 5–10 ml liposome suspension) and a final change of PBS. Fresh dialysis casings were used with each buffer change as these casings became occluded with adriamycin. Chromatography of the dialyzed vesicle preparations on G-75 Sephadex columns (1 × 30 cm) showed that greater than 95% of the adriamycin in the preparation was encapsulated in the vesicles.

Serum incubation studies in vitro

Dialyzed vesicles (60 μm/ml) containing adriamycin were diluted 1:3 with heat inactivated, filtered fetal calf serum (FCS) (GIBCO, Grand Island, NY) and incubated at 37°C for 1, 6 or 24 hr. Adriamycin retention in vesicles was determined spectrophotometrically (O.D. 480 nm) after separation of vesicles from serum on Sepharose 6B columns (1 × 50 cm).

Liposome size distribution

The size distribution of liposomes before and after extrusion through Nucleopore membranes and after 24 hr serum incubation was determined by negative-stain electron microscopy, as previously described [13], on silica monoxide coated grids [14].

Cultured chick heart cells

Hearts removed from embryonic chickens on the 9th or 10th days of incubation were rinsed in Ca^{2+}, Mg^{2+} free Hanks balanced salt solution to remove blood, minced finely and incubated for 1 hr at 37°C with Enzar-T crystallized trypsin (Armour Pharmaceuticals, Phoenix, AZ) before dissociation by repeated pipetting in medium. The cell suspensions were filtered through 200 gauge mesh to remove cell clumps and the cell concentration determined using a Fuchs–Rosenthal hemocytometer. Cells were cultured in low K^+ media (to promote heart cell beating) containing 10% horse serum, as described by De Haan [15]. Cells (5×10^5) were added in 1 ml medium to a 35 mm plastic tissue culture dish containing a circular glass coverslip. After 24 hr of culture the medium was changed with warmed medium and the time for 10 contractions for each of 10 contracting areas in each dish was determined after 1 hr of further incubation. The medium was then changed in all dishes containing beating cells with either warm control or treatment media and the beating rates determined after 5 min, 1 hr and 4 hr. All microscopical observations were carried out using a 37°C stage.

Toxicity of plain liposmes, and free and liposome-encapsulated adriamycin

Groups of 4–8 C57/B1 6 female mice were administered either free adriamycin or liposome-encapsulated adriamycin in doses ranging from 10 to 50 mg/kg body weight via tail vein injection. Groups of 4–8 Swiss female mice were injected with plain liposomes, with the doses ranging from 0.5 to 10 g/kg. Animals were observed up to 120 days post drug administration and acute and chronic toxicity determined.

Electron microscopic examination of cardiac tissue

Groups of 3 C57/Bl 6 mice received, via tail vein injection, either 20 or 40 mg/kg adriamycin as free drug or encapsulated in liposomes. All groups were killed 6 days after injection. Atria and ventricles were separated from dissected hearts and minced on a glass plate at 0°C. Tissues were fixed sequentially in 3%

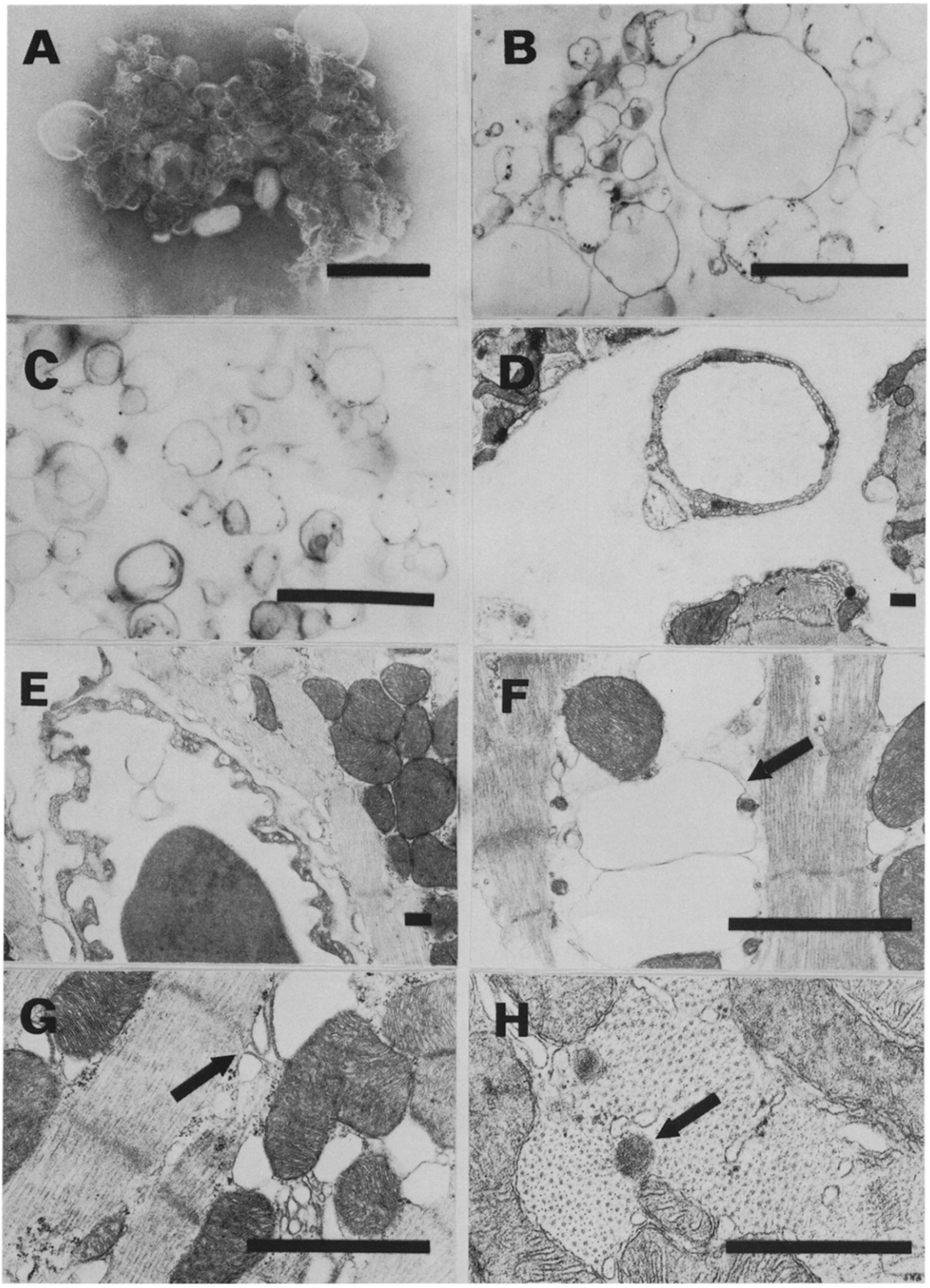

Fig. 1. *Electron photomicrographs. All magnification bars* = 0.5 μm. (A) *Liposomes prepared with high initial adriamycin/lipid ratio* (1/5); *negative stain preparation.* (B) *Liposomes prepared with low initial adriamycin/lipid ratio* (1/20). *Agar embedding prior to sectioning.* (C) *Preparation as in* B *following sequential extrusion through* 0.4 *and* 0.2 μm *pore membranes.* (D) *Capillary region in atrium of animal* 6 days *after receiving* 40 mg/kg *adriamycin of free drug. Note large increase in perivascular space.* (E) *Capillary region in atrium of animal* 6 days *after receiving* 40 mg/kg *adriamycin encapsulated in liposomes. Only slight enlargement of perivascular space is seen.* (F) *Markedly dilated sacroplasmic reticulum* (*arrow*) *or ventricule tissue* 6 days *after receiving* 40 mg/kg *adriamycin as free drug.* (G) *Sarcoplasmic reticulum* (*arrow*) *in ventricular tissue* 6 days *after receiving* 40 mg/kg *adriamycin in liposomes.* (H) *Electron-dense body* (*arrow*) *in atreal tissue* 6 *days after receiving* 40 mg/kg *adriamycin as free drug.*

glutaraldehyde, 2% OsO_4 and 0.5% aqueous uranyl acetate prior to embedding in Epon 812. Thin sections (700 Å) were stained with uranyl acetate and lead citrate, and examined with a Siemens 101 electron microscope.

A total of six specimens were examined from each tissue treatment group. After surveying each specimen, two representative photographs were taken of capillary, myocyte plasma membrane, nucleus, myofibril and mitochondrial–sarcoplasmic reticulum regions. Photographs were grouped by tissue (atrium or ventricle) and region photographed, and compared with control tissue to identify abnormalities. Three abnormalities were identified: relative increase in perivascular space, presence of electron-dense bodies and enlargement of the sarcoplasmic reticulum. Photographs were then placed in random sequence within each group and abnormalities graded from 0 (no abnormality) to 4+ (severe abnormality) without knowledge of treatment. Finally, photographs were identified as to treatment group and results tabulated as a numerical average.

Tissue distribution of adriamycin

Mice were injected i.v. with 20 mg/kg free or liposome entrapped adriamycin. Mice were sacrificed at 30 min, 1, 2, 4, 8, 12, 24 and 48 hr. Blood was removed by cardiac puncture and plasma prepared and frozen for subsequent analysis. Hearts were removed rapidly and frozen for subsequent analysis. Tissues were assayed for adriamycin using the fluorescence method described by Schwartz [16], which determines adriamycin and some metabolites.

Therapeutic comparison of free adriamycin and adriamycin encapsulated in liposomes

Groups of DBA 2/J female mice were injected intraperitoneally with 10^6 L1210 leukemia cells. Twenty-four hours later, groups received either PBS, free adriamycin or adriamycin encapsulated in liposomes via tail vein injection. Animals were observed for 45 days post injection and the day of death determined.

RESULTS

In vitro *and* in vivo *toxicity of non-drug-containing liposomes*

Table 1 shows that plain liposomes bearing a net positive charge (SA) were more toxic than neutral or positively charged liposomes to cultured chick heart cells. At 10^{-4} M, positively charged liposomes immediately inhibited myocardial cell contraction rate (MCCR), whereas neutral (PC) and negatively charged (PG) liposomes only reduced MCCR up to 4 hr to 20–80% of control levels.

Similarly, the data in Table 2 indicate that positively charged liposomes were 6 times more toxic than neutral or negatively charged vesicles against normal Swiss mice.

Standardization of adriamycin liposomes

Negatively charged liposomes were utilized in subsequent experiments because they were found to be less toxic than positively charged liposomes and tended to remain in suspension longer than neutral liposomes.

In a preliminary experiment, adriamycin was encapsulated (PG:PC:Chol, 1:4:5 liposomes) at an initial adriamycin: total lipid molar ratio of 1:5. It was noticed, however, that this preparation was difficult to maintain in suspension and that attempts to separate bound from free adriamycin by filtration on G-75 Sephadex resulted in retention of liposomes on the gel bed. Negative-stain electron photomicrographs of this preparation (Fig. 1A) showed that liposomes prepared with high initial adriamycin:lipid ratio (1:5) resulted in the production of large, poorly defined aggregates. The adriamycin content was gradually reduced in subsequent preparations until these aggregates

Table 1. In vitro *toxicities of plain liposomes*

Preparation	MCCR as percentage of controls 5 min	1 hr	4 hr
Positive liposomes* (SA:PC:Chol; 1:4:5)	Immediate cessation of beating		
Negative liposomes* (PG:PC:Chol; 1:4:5)	100	87	80
Uncharged liposomes* (PC:Chol; 1:1)	100	84	76

*Liposomes added at final concentration of 10^{-4} M lipid.
Chick myocardial contraction rate (MCCR) after incubation of uncharged and positively and negatively charged liposome preparations.

were no longer observed. This occurred at an initial adriamycin:lipid ratio of 1:20. Immediate separation of this preparation on G-75 Sephadex columns removed 20% of the adriamycin, while subsequent dialysis over 24 hr at 37°C removed a further 25%, resulting in a liposomal adriamycin preparation of final adriamycin:lipid ratio of approximately 1:30. The unfiltered preparations and that following extrusion through 0.2 μm membranes are shown following agar embedding and sectioning in Figs. 1B and 1C respectively.

Incorporation of adriamycin into liposomes markedly reduced the mean size of vesicles both prior to and after extrusion (Fig. 2). Unextruded liposomes made without adriamycin had a mean vesicle diameter of 2.4 μm, while those with adriamycin had a mean diameter of 0.26 μm. Extrusion through 0.2 μm pore membranes reduced the mean diameter of liposomes without adriamycin to 0.24 μm and those with adriamycin to 0.15 μm. Incubation with FCS for 24 hr did not greatly alter the size distribution of vesicles ($\bar{x} = 0.12$ μm).

Table 3 shows the serum stability of freshly prepared, dialyzed, extruded (0.2 μm) adriamycin liposomes. Liposomal retention of adriamycin was 85% at 24 hr. Addition of a higher concentration of serum (1:1) or a more dilute suspension of liposomes (20 μM lipid/ml) did not alter liposomal retention of adriamycin. Table 3 also shows that storage of vesicles under N_2 at 4°C for 6 days prior to addition to serum resulted in poorer adriamycin retention (65% at 24 hr). Based on these findings, the liposomes encapsulating adriamycin used in all subsequent experiments were freshly prepared, composed of PG:PC:Chol (1:4:5), extruded through 0.2 μm pore membranes and had a final adriamycin: lipid ratio of approximately 1:30.

In vitro *and* in vivo *toxicity of adriamycin liposomes*

Liposomal adriamycin was significantly less toxic in terms of reduced myocardial cell contraction rate (MCCR) than free drug after a 4 hr incubation. At 10^{-5} M, free adriamycin inhibited MCCR by 46% compared to 18% for liposomal adriamycin, and at 10^{-6} M free adriamycin inhibited MCCR by 20% compared to 9% for liposomal adriamycin.

Administration of free adriamycin in single i.v. doses of 10–50 mg/kg produced a characteristic pattern of short- (4–14 day) and long- (approx. 45–70 day) term death. Thus, the LD_{50}s (Table 4) were calculated as short- and long-term groups. Administering adriamycin in liposomes (up to 50 mg/kg) did not alter the

Table 2. Toxicities of plain MLV liposomes in mice

Composition	Molar ratio	Approximate LD_{50} (g/kg)
SA/PC/Chol	1:4:5	1.1
PC/Chol	1:1	7.2
PG/PC/Chol	1:4:5	7.5

SA = Stearylamine; PG = phosphatidylglycerol; PC = phosphatidyl choline; Chol = cholesterol.

Table 3. Effect of serum incubation on adriamycin encapsulation

	% Adriamycin retained in vesicles after incubation for indicated times		
	1 hr	6 hr	24 hr
Fresh vesicles	94	91	85
Vesicles after storage for 6 days at 4°C (no serum present)	90	84	65

Dialyzed vesicles (60 μM/ml) were diluted 1:3 with fetal calf serum and incubated at 37°C for the indicated times. Adriamycin retention was determined after separation of vesicles from serum on Sepharose 6B columns.

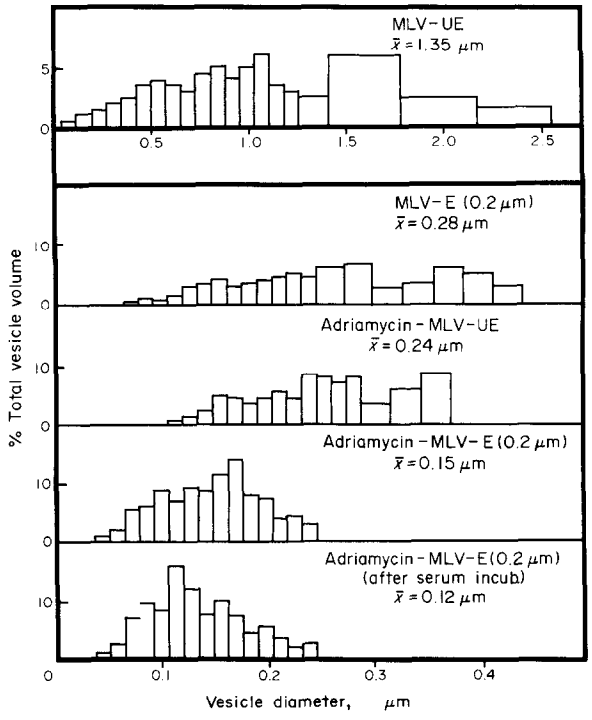

Fig. 2. Size distribution of liposome preparations. Size distribution of liposomes (PG:PC:Chol—1:4:5) was determined by negative-stain electron microscopy before (UE) and after extrusion through 0.2 μm pore size filters (E, 0.2 μm). Mean size determinations are an encapsulated volume average.

pattern of short- and long-term lethality, but did substantially reduce overall toxicity.

Table 5 shows the effects of free and liposome-encapsulated adriamycin on cardiac tissue morphology 6 days post drug administration. These effects differed in atrium and ventricle. Increased size of perivascular space was most pronounced in the atrium of animals receiving free adriamycin (Fig. 1D), whereas administration of encapsulated adriamycin resulted in less swelling (Fig. 1E). Dilation of the sarcoplasmic reticulum was most prominent in ventricular tissue of animals receiving free adriamycin (Fig. 1F), while encapsulated adriamycin resulted in less dilation (Fig. 1G). The presence of electron dense bodies was seen in atrial (Fig. 1H) and ventricular tissues following free adriamycin administration. These bodies were not observed following administration of encapsulated adriamycin or in control tissue.

Tissue distribution of adriamycin

Figure 3 shows that following equidosing (at 20 mg/kg), both initial rapid-phase (0–2 hr) and subsequent slow-phase (2–24 hr) plasma clearance of adriamycin was markedly slowed in animals receiving liposome-encapsulated as compared with free drug. By 48 hr post drug administration, however, the plasma levels of adriamycin were similar (0.5 μg/ml) in both treatment groups.

Figure 4 shows that following free adriamycin administration, peak uptake by the heart occurred at 2–4 hr, resulting in tissue levels (approximately 25 μg/g) 5-fold greater than in liposome-treated mice. Integrating tissue concentrations over the first 48 hr post drug administration showed that cardiac content of adriamycin in liposome-treated mice averaged 2.7 μg/g, as compared with 7.1 μg/g following free drug administration.

Table 4. Toxicity of adriamycin in DBA 2mice

Toxicity	Dosage form Free	MLV
Short-term*	20 mg/kg	Approx. 50 mg
Long-term†	10–15 mg/kg	25–30 mg/kg

*Deaths within 14 days of injection.

†Deaths among mice surviving greater than 14 days after injection (death usually occurred at 45–70 days).

LD_{50} calculated from survival curves of groups of mice injected i.v. with 10–50 mg/kg adriamycin i.v. in a single injection.

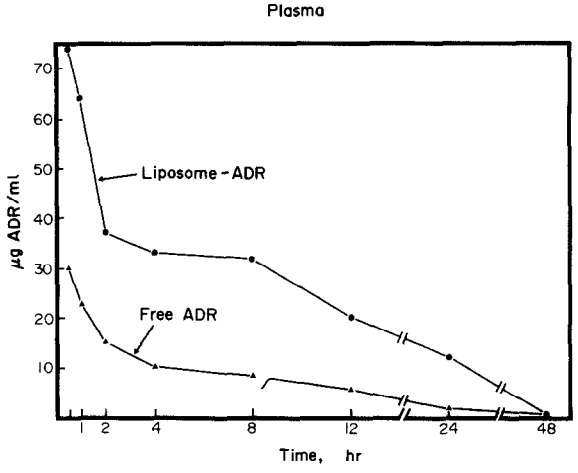

Fig. 3. Plasma adriamycin levels. Plasma adriamycin levels were determined by fluoroscopy up to 48 hr *after animals received either free (▲—▲) or liposome-encapsulated (●—●) drug. Each point represents the mean plasma level (in μg adriamycin/*ml *plasma) of 3 animals.*

Table 5. Morphologic changes in mouse heart after treatment with adriamycin

Tissue	Form/drug dose (mg/kg)	Tissue abnormality: Increased perivascular space	Enlarged sarcoplasmic reticulum	Electron dense bodies
Atrium	Free 20	+++	++	+
	Free 40	+++	++	+
	Liposome 20	++	+	–
	Liposome 40	+	–	–
Ventricle	Free 20	++	++	–
	Free 40	+	+++	–
	Liposome 20	+	+	–
	Liposome 40	–	++	–

Animals received either free or liposome-encapsulated adriamycin at 20 or 40 mg/kg and were killed 6 days post drug administration. Tissue abnormalities were scored on a scale of 0 (no abnormality) to 4+ (severe abnormality). Further details are described in the Methods section.

The determinations for liposome adriamycin in the present experiments give net adriamycin concentrations, which are comprised of free plus entrapped drug. The data from Table 3 suggest that at early times after injection most of the adriamycin is in liposomes, whereas at later times significant amounts of free drug would be present.

Anti-tumor activity of free and encapsulated adriamycin

Table 6 shows that at 10 mg/kg, free or entrapped adriamycin increased survival times to a similar extent, compared with controls. At 20 mg/kg, early deaths were evident due to toxicity for the free drug, whereas treatment with liposome-entrapped adriamycin was not toxic and resulted in increased survival time compared to controls. The effects with adriamycin liposomes at 20 or 10 mg/kg were similar.

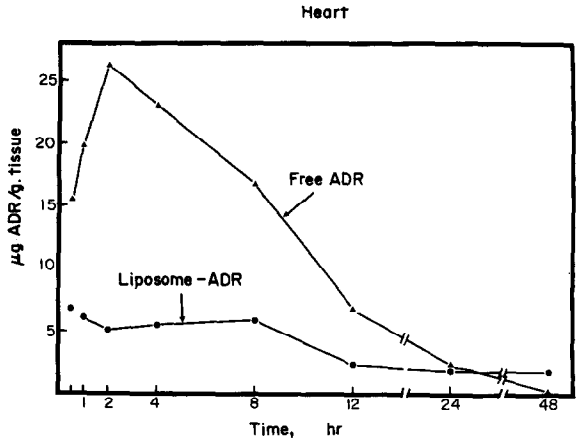

Fig. 4. Heart adriamycin levels. Heart adriamycin levels were determined by fluoroscopy up to 48 hr after animals received either free (▲—▲) or liposome-encapsulated (●—●) drug. Each point represents the mean heart level (in μg adriamycin/gm tissue) of 3 animals.

DISCUSSION

Our method of producing adriamycin liposomes yields vesicles that have well defined, uniform, reproducible characteristics. They have a mean diameter of 0.15 μm, are largely unilamellar (Fig. 1C), do not release adriamycin to PBS and are stable in serum (15% adriamycin release over 24 hr). Several lines of reasoning suggest that the majority of encapsulated adriamycin is located at the lipid/aqueous interface of the internal lamellar surface. Firstly, it has been shown by fluorometric spectra analysis [17] that adriamycin preferentially associates with the lipid/aqueous interface of liposome membrane lamella. Secondly, in the present study, following column chromatography that removes free adriamycin in solution external to the liposome, a further quantity of adriamycin is slowly released during exhaustive dialysis and probably represents drug that is bound to the external lamellar surface. Adriamycin does not appear to be able to cross the lipid bilayer, as remaining drug is not removed by further dialysis. Thirdly, the quantity of adriamycin that is retained is 10-fold greater than that which could be contained in solution in the internal aqueous space (based on a capture volume of 3 μl/μM lipid [11], an aqueous adriamycin solubility of 1 μM/ml) and therefore must be mainly complexed with the internal lamellar surface.

The quantity of adriamycin retained in our liposome preparation was relatively low (1:30 adriamycin:lipid) and similar to that used by Forssen and Tokes [9]. Use of positively charged vesicles containing therapeutic levels of adriamycin at this encapsulation efficiency (5–20 mg/kg requiring 300–1200 μm lipid/kg) is precluded by the inherent toxicities of these liposomes *in vitro* (Table 1) and *in vivo* (Table 2).

A variety of animals, including rabbits, rats and mice, have been used as models of human adriamycin cardiotoxicity (for review see [18]). In rabbits, as in humans, adriamycin toxicity is

*Table 6. Effect of adriamycin on L1210 leukemia**

Dose of adriamycin	Mean survival time* [Days ± S.E. (number of mice surviving†/total mice per group)] Free adriamycin	Adriamycin liposomes
20 mg/kg	9.1 ± 1.1 (0/10)	20.3 ± 7.3 (3/17)
10 mg/kg	19.5 ± 4.6 (4/20)	21.6 ± 7.9 (4/19)
Control	7.9 ± 1.3 (0/21)	—

*Mice injected 10^6 L1210 tumor i.p. day 0, treated i.v. 24 hr later.
†Mice surviving more than 40 days.

cumulative and associated with impaired left ventricular performance, histopathologic cardiac lesions and biochemical abnormalities characteristic of congestive heart failure [18]. It is known that in the rat and mouse [19, 20] adriamycin causes cumulative histologically demonstrable cardiotoxicity, but it has not been conclusively demonstrated that heart failure is the cause of death. The mouse, however, remains a desirable model for evaluating adriamycin, because the availability of specific mouse tumor lines allows the determination of both toxicity and therapeutic effect in the same organism.

In the present study, adriamycin-induced cardiac cell damage demonstrated *in vitro* and cardiac tissue damage *in vivo* (Table 5) was reduced by encapsulating the drug in liposomes. Encapsulating adriamycin reduced cardiac uptake of the drug 2-fold and was associated with a 2-fold increase in LD_{50}.

Two previous studies of the tissue distribution of adriamycin encapsulated in anionic liposomes differ in the reported cardiac uptake of adriamycin. Rahman *et al.* [10] showed cardiac uptake of encapsulated adriamycin to be greater than that of free drug, while Forssen and Tokes [9] reported a reduced uptake of encapsulated adriamycin. We think that this difference was due to the composition of the respective anionic liposome preparations. Our attempts to construct vesicles similar to those of Rahman *et al.*, with a high adriamycin lipid ratio (greater than 1:20), resulted in the production of ill-defined adriamycin–lipid aggregates (Fig. 1A). This preparation continuously released drug during exhaustive dialysis if dialysis casings (which become occluded with adriamycin) were changed with each change of buffer. When adriamycin content was reduced to levels that permitted production of typical vesicle structures (adriamycin:lipid ratio of less than 1:30), reduced cardiac uptake of drug was noted.

No increase in therapeutic effect was associated with administering encapsulated adriamycin. Several studies have demonstrated the enhanced cytotoxicity of liposome-encapsulated drugs *in vitro* (for review see [21]). It has been proposed that this effect is due to specific liposome-mediated drug uptake by cells: negatively charged liposomes via fusion with malignant cells and neutral or positively charged liposomes via endocytosis [22]. It seems unlikely, however, that such liposome–tumor cell interactions occur *in vivo*, where intravenously administered liposomes would be mainly confined to the circulatory system or removed by cells of the reticulo-endothelial system. Thus, we think that it is probable that the antitumor effects of adriamycin liposomes are probably due to slow release of the drug in the free form from the liposomes, as has been suggested for other drug–liposome preparations [12, 21]. As the antitumor effects of liposome adriamycin are similar at equal doses (such as 10 mg/kg) where acute toxicity is not observed, this suggests that equal amounts of the drug are able to reach the target tumor cells.

Thus, it can be concluded that carefully defined adriamycin–liposome preparations significantly reduce toxicity *in vivo* and *in vitro* compared to free drug. It appears that the reduction in toxicity is related to a reduction in cardiotoxicity. The therapeutic efficacy of the liposome-entrapped drug is not reduced compared to free drug. These conclusions suggest that as total dose limitation in humans is related to cardiotoxicity, even a modest two-fold decrease in toxicity could enable an increase in the total dose of adriamycin administered or a reduction in the likelihood of toxicity.

Acknowledgements—We thank Dr. R. F. Zeigel for helpful discussions and D. Milholland, R. Lazo, J. Goranson and G. Johnson for technical assistance.

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