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Liposomes with entrapped doxorubicin exhibit extended blood residence times

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The blood residence time of liposomes with entrapped doxorubicin is shown to be significantly longer than for identically prepared empty liposomes. Liposomal doxorubicin systems with a drug-to-lipid ratio of 0.2 (w/w) were administered at a dose of 100 mg lipid/kg. Both doxorubicin and liposomal lipid were quantified in order to assess in vivo stability and blood residence times. For empty vesicles composed of phosphatidylcholine (PC)/cholesterol (55:45, mole ratio) and sized through filters of 100 nm pore size, 15–25% of the administered lipid dose was recovered in the blood 24 h after i.v. injection. The percentage of the dose retained in the circulation at 24 h increased 2–3-fold when the liposomes contain entrapped doxorubicin. For 100 nm distearoyl PC/chol liposomal doxorubicin systems, as much as 80% of the injected dose of lipid and drug remain within the blood compartment 24 h after i.v. administration.

Introduction

Applications for liposomes as drug carriers are becoming apparent. In particular, acute and chronic toxicities associated with certain drugs can be reduced if the agent is presented in association with liposomes. This reduced toxicity is accompanied by maintained or enhanced efficacy. Therefore, the liposomal carrier can provide a significant improvement in the therapeutic index of the entrapped drug. Two such formulations, an amphotericin B-lipid complex and liposomal doxorubicin, are currently being evaluated in human clinical trials [1–3].

Many investigations have documented the potential therapeutic benefit of liposomal doxorubicin, however, the mechanism(s) underlying the biological activity of these preparations are not clear. We [4] and others [5] have demonstrated in animal models that the biological activity of liposomal doxorubicin can be modulated by alterations in vesicle lipid composition and size. Liposomal doxorubicin systems formulated with saturated phospholipids species (such as dipalmitoyl- or distearoyl-PC) and cholesterol are the least toxic and antitumour activity increases as vesicle size decreases. It is also well established that liposome size and lipid composition can dramatically alter the clearance kinetics of liposomes [6–8]. In particular, liposomes prepared with saturated phospholipid species and cholesterol exhibit superior drug retention in vivo than liposomes composed of unsaturated phospholipids [7]. Further, as vesicle size is decreased there is a concomitant increase in blood residence times [8]. In this study we demonstrate that the blood residence time is influenced not only by the physical characteristics of the vesicle but also by the activity of the entrapped agent, doxorubicin. This effect is important for understanding the mechanisms determining the biological activity of the encapsulated drug.

Abbreviations: MLV, multilamellar vesicle; LUV, large unilamellar vesicle; SUV small unilamellar vesicle; egg PC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; chol, cholesterol; RES, reticuloendothelial system; QELS, quasielastic light scattering; i.v., intravenous.

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Materials and Methods

Animals. Female DBA/2J mice 6–8 weeks old were obtained from Jackson Animal Laboratories (Califonia). Groups of four mice per experimental point were given the specified treatment as a single i.v. dose via the

lateral tail vein. The dose, administered in a volume of 200 μl, was based on mean body weight. Blood was collected from the carotid artery or through heart puncture and was placed in EDTA-treated microtainers (Becton-Dickinson, Canada). Plasma was prepared by centrifuging (200 × *g*) blood samples for 10 min in a clinical centrifuge. Total plasma volume per animal was taken to be 4.55% of mean body weight. Control blood samples containing known amounts of liposomes showed that negligible amounts of the liposomal lipid was associated with the pelleted blood cells. The recovery of liposomes was similar if determined from whole blood or from plasma.

Liposome preparation. Egg phosphatidylcholine (egg PC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol was obtained from Sigma Chemicals (St. Louis. MO), and all other chemicals were of reagent grade. Lipid mixtures were prepared in a chloroform solution and subsequently were dried under a stream of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2 h. Multilamellar vesicles (100 mg lipid/ml) were formed by hydrating the dried lipid with 300 mM citric acid (pH 4.0). The resulting preparation was frozen and thawed five times and subsequently extruded employing an extrusion device (Lipex Biomembranes, Vancouver, Canada) ten times through two stacked polycarbonate filters (Nuclepore, Canada) of the indicated pore size according to standard procedures [9,10]. When DSPC was employed the sample was extruded at 65 °C employing a thermobarrel extrusion device. Liposome particle size was determined by quasi-elastic light scattering measurements (employing a Nicomp 370 particle sizer, operating at a wavelength of 632.8 nm).

Doxorubicin encapsulation. Doxorubicin, obtained from Adria Laboratories (Mississauga, Canada), was encapsulated in liposomes in response to transmembrane pH gradients as described previously [11,12]. Briefly, the pH of the liposome suspension initially at pH 4.0, was raised to pH 8.0–8.5 with 0.5 M Na_2CO_3. The liposome preparation was subsequently heated to 60 °C for 5 min. These liposomes were then transferred to a preheated (60 °C) vial of doxorubicin, typically adding enough lipid to achieve a final drug-to-lipid ratio of 0.2 (w/w). This mixture was incubated with periodic mixing for 10 min at 60 °C. Doxorubicin concentration, lipid concentration and encapsulation efficiency were determined as described previously [12]. The procedure resulted in entrapment efficiencies in excess of 95%. Solutions for injection were prepared with sterile physiological saline such that the specified dose could be delivered in 200 μl.

Quantitation of liposomal lipid and doxorubicin. Liposomal lipid was quantified employing the lipid marker [^{3}H]cholesterol hexadecyl ether (NEN, Canada). This lipid marker does not readily exchange with lipoproteins, is not subject to esterase activity, and remains associated with cells once internalized [13,14]. Due to the long circulation times observed for the liposomes employed in this report, the extent of [^{3}H]cholesterol hexadecyl ether exchange into lipoproteins was examined for the egg PC/chol and DSPC/chol liposomes to ensure that exchange effects did not bias the results. Vesicles were isolated from lipoproteins according to the procedure described by Scherphof et al. [15]. Approximately 1 ml of mouse plasma containing vesicles was applied to a Bio-Gel A-15m gel filtration column (30 × 1.5 cm) which was eluted at 4 °C and a flow rate of 6 ml/min using Tris-buffered saline (pH 7.4). 80 fractions were collected and analyzed for radioactivity. Vesicles elute in the void volume and are well separated from HDL particles, which are the lipoprotein fraction known to interact with lipid vesicles [15]. Separation of vesicles from HDL was demonstrated by calibrating the gel filtration column by eluting various lipoprotein fractions isolated using standard ultracentrifugation procedures. [^{3}H]Cholesterol labelled HDL was shown to be included in the gel. Moreover, fasted mouse plasma prelabelled with [^{3}H]cholesterol gave an elution profile in which the vesicle peak was clearly resolved from the HDL peak. Plasma samples collected at 4 h and 24 h following injection of [^{3}H]cholesterol hexadecyl ether labelled liposomes into mice showed no incorporation of radioactivity into the HDL peak.

For scintillation counting, 50–100 μl plasma was added to 400 μl of methanol followed by 100 μl of hydrogen peroxide. Hydrogen peroxide was added to all plasma samples due to the varying degree of hemolysis obtained during collection and preparation of the blood samples. Subsequently, 5 ml Pico-Fluor 40 (Packard, Canada) scintillation cocktail was added. Samples were left for at least 24 h prior to couting in a Beckman model LS 3801 scintillation counter.

Doxorubicin was determined employing a fluorescent assay procedure. Plasma (50–100 μl) was diluted to 1 ml in physiological saline (adjusted to pH 4.0 with HCl). Subsequently, the sample was extracted with 2 ml of chloroform/isopropyl alcohol (1 : 1, v/v). Following vigorous mixing and a brief centrifugation, the organic phase was collected. The fluorescence of this phase was determined (excitation wavelength of 500 nm and emission wavelength 550 nm) employing a Shimadzu RF-540 spectrofluorometer. A standard doxorubicin curve was prepared employing a similar extraction procedure. Where necessary, plasma samples were diluted such that the doxorubicin level within the 1 ml acidic saline solution fell within the range of the standard curve. All values are represented as μg doxorubicin fluorescent equivalents per 100 μl plasma. HPLC analysis of plasma extracts obtained from the 24 h time point of animals

receiving liposomal doxorubicin systems indicated that more than 98% of the fluorescence detected was due to non-metabolized doxorubicin.

Results

The antitumour activity of liposomal doxorubicin in the L1210 lymphocytic leukemia model is sensitive to vesicle size [4] where greater efficacy is observed for smaller systems. Vesicle size also influences dramatically the blood clearance kinetics of empty liposomes [8,16]. This effect is illustrated in Fig. 1 for liposomes composed of DPPC/chol (55 : 45, mol/mol). Vesicles sized through filters with 1.0 μm pore size (Fig. 1A, open symbols) have a mean diameter of approx. ~ 1.2 μm as determined by QELS. Within one hour following i.v. injection more than 90% of these large vesicles have been removed from the circulation. In contrast, vesicles sized through filters with 0.1 μm pore size (Fig. 1B, open symbols), exhibiting a mean diameter of 0.12 μm, are retained in the circulation for extended periods. These vesicles have a circulation half-life of approx. 8 h

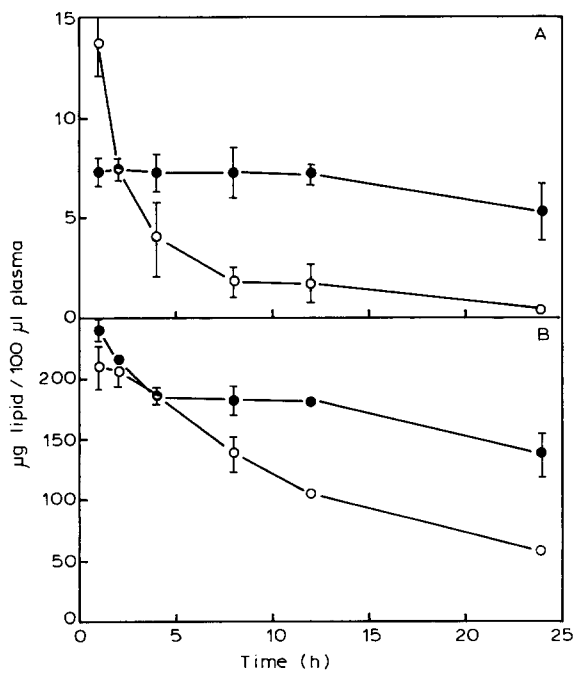

Fig. 1. Liposomes (open symbols) and liposomes loaded with doxorubicin (solid symbols) employing ΔpH driven uptake were injected into DBA/2J mice at a doxorubicin dose of 20 mg/kg or a lipid dose of 100 mg/kg. Vesicles (DPPC/chol, 55 : 45) prepared as indicated in Methods exhibited mean diameter of 1.2 μm (Fig. 1A) or 0.12 μm (Fig. 1B). Blood was collected at the indicated time points in microtainers containing EDTA. The blood samples were centrifuged for 10 min at 500 × g, subsequently the plasma was removed and prepared for assays. Lipid was quantified employing the radiolabel trace lipid [^{3}H]cholesterol hexadecyl ether as indicated in the Methods. Each point represents an average of four animals and the error bars reflect the standard deviation.

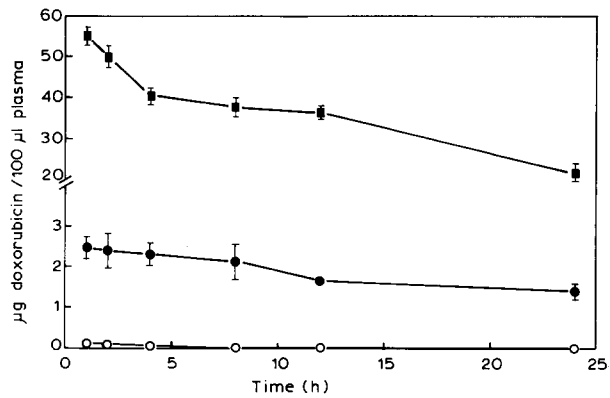

Fig. 2. The level of doxorubicin recovered in the plasma of mice treated with either free doxorubicin (open circles), 1.2 μm DPPC/chol liposomal doxorubicin (solid circles) or 0.12 μm DPPC/chol liposomal doxorubicin (solid squares). Doxorubicin was determined employing the fluorescent assay procedure described in the Methods. Samples diluted to 1 ml in acidic saline were extracted with 2 ml of chloroform isopropylalcohol (1 : 1). The organic phase was isolated and fluorescence was determined. Each point represents an average of four animals and the error bars indicate the standard deviation.

and almost 20% of the injected lipid dose is still circulating at 24 h.

It is generally assumed that liposome elimination from the circulation occurs as a result of vesicle uptake by phagocytic cells of the reticuloendothelial system (RES) [17]. Since doxorubicin is a potent cytotoxic agent, it would be expected that cells which accumulate liposomal doxorubicin may be affected by the presence of the entrapped drug. It is therefore important to determine whether encapsulated doxorubicin can alter the clearance behaviour of the liposomal carrier. The data is Figs. 1A and 1B (see closed symbols) clearly demonstrate that entrapped doxorubicin extends the circulation times of the associated vesicles. Regardless of vesicle size, this results in a 2–10-fold increase in the amount of liposomal lipid within the circulation at time points beyond 8 h. It is interesting that the clearance rates of 0.12 μm empty vesicles and 0.12 μm liposomal doxorubicin systems are similar for the first 4 h following i.v. administration.

Circulating doxorubicin concentrations in animals injected with free drug are 500- and 25-fold lower (at time points beyond 4 h) than obtained when the drug is encapsulated in 0.12 μm and 1.2 μm vesicles, respectively (Fig. 2). It should be emphasized that no attempt has been made to differentiate between the level of free drug and liposome associated drug in animals given liposomal doxorubicin. However, since the free drug is cleared very rapidly from the plasma, with an initial circulating half-life of less than 5 min [4], it is reasonable to assume that the large majority (> 95%) of doxorubicin measured in the plasma of animals receiving liposomally entrapped doxorubicin is associated with the liposomal carrier. The drug-to-lipid ratio obtained

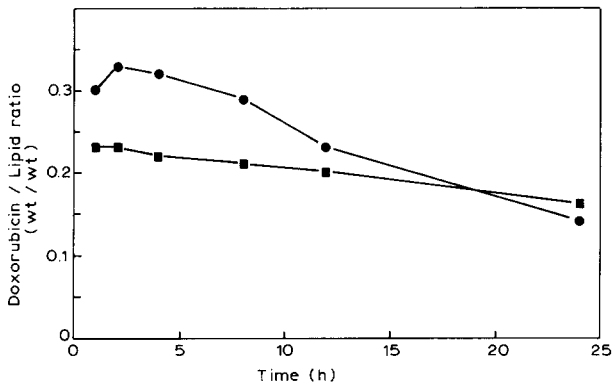

Fig. 3. Drug-to-lipid ratio determined in the plasma of mice treated with DPPC/chol liposomal doxorubicin sized through 0.1 μm (solid squares) and 1.0 μm (solid circles) filters. Lipid levels were determined as indicated in Fig. 1. Doxorubicin was determined as indicated in Fig. 2.

in plasma samples of animals treated with DPPC/chol liposomal doxorubicin (Fig. 3) supports this assumption and indicates that this liposomal formulation is extremely stable. For the 0.12 μm diameter vesicle system the drug-to-lipid ratio (w/w) prior to injection was determined to be 0.22. The plasma samples obtained 1 h after administration indicated a drug-to-lipid ratio (w/w) of 0.22 and this dropped linearly to a ratio of approximately 0.16 at 24 h. The drug-to-lipid ratio profile obtained from animals given 1.2 μm diameter DPPC/chol liposomal doxorubicin indicates an apparent increase in drug-to-lipid ratio for several hours after injection. This apparent increase in drug-to-lipid ratio may reflect sample heterogeneity with respect to size and drug-to-lipid ratio.

The data presented thus far were obtained for vesicles composed of DPPC/chol (55 : 45). However, as indicated in Fig. 4, the increased circulation time exhibited by these liposomal doxorubicin systems was observed

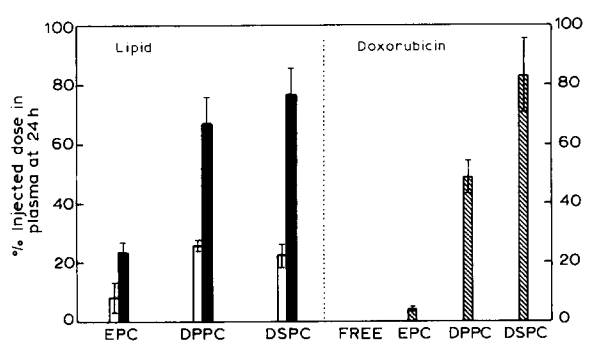

Fig. 4. The percentage of injected dose recovered in plasma 24 h after i.v. injection of doxorubicin (hatched bars) and liposomes (solid bar: liposomal doxorubicin and open bar: empty liposomes). The vesicles were prepared with the indicated phosphatidylcholine species and cholesterol (55 : 45) and sized through filters with 0.1 μm pore size. The recovery of free drug in plasma at 24 h was less than 0.1% but well within the detection limits of doxorubicin assay employed (see Methods).

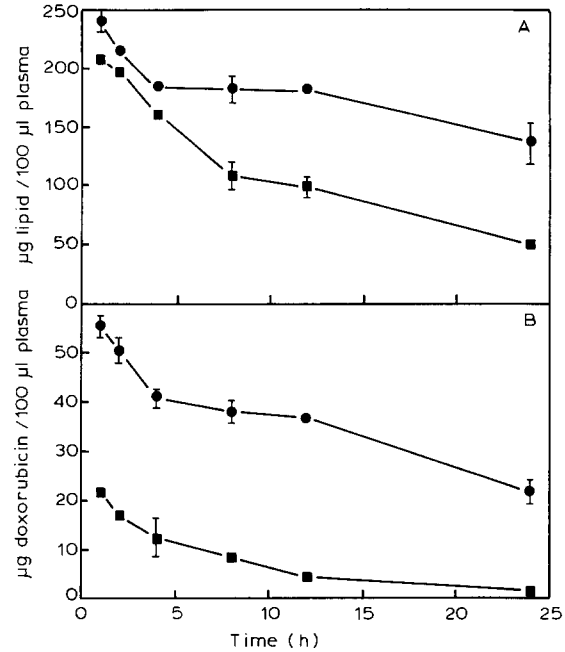

Fig. 5. Liposomal lipid (Fig. 5A) and doxorubicin (Fig. 5B) levels obtained in plasma of mice injected with 0.12 μm DPPC/chol liposomal doxorubicin (solid circles) or 0.12 μm egg PC/chol liposomal doxorubicin (solid squares). Doxorubicin and lipid were quantified as indicated in Figs. 1, 2 and Methods. Each point represents the average of four animals and the error bars indicate the standard deviation.

for a number of lipid compositions. For example, 80% of the injected lipid dose and injected doxorubicin dose remain in the circulation 24 h after injection of liposomal doxorubicin systems prepared with vesicles composed of DSPC/chol (55 : 45) and sized through the 0.1 μm pore size filters. As much as 10% of the injected doxorubicin was still circulating three days after administration (results not shown). The data in Fig. 4 also indicates the influence of lipid composition on doxorubicin release. Liposomal doxorubicin systems prepared with 0.12 μm vesicles composed of DSPC/chol showed no decrease in the initial drug-to-lipid ratio 24 h after i.v. administration. In contrast, DPPC/chol and egg PC/chol vesicles display a decrease in drug-to-lipid ratio at 24 h of 27% and 80%, respectively. This effect is illustrated in greater detail in Figs. 5 and 6 for egg PC/chol and DPPC/chol liposomal doxorubicin systems. Quantitation of liposomal lipid indicate that the initial rates (for the first 4 h) of elimination for egg PC/chol and DPPC/chol 0.12 μm liposome are similar (Fig. 5A). However, at times beyond 8 h the level of lipid found in the plasma is 2-fold less for animals given egg PC/chol liposomes. Although the initial rates of liposome clearance are comparable, 3-fold less doxorubicin is found in the plasma of animals given egg PC/chol liposomal doxorubicin (Fig. 5B). The differences in plasma doxorubicin concentrations become more pronounced at time points in excess of 8 h. As

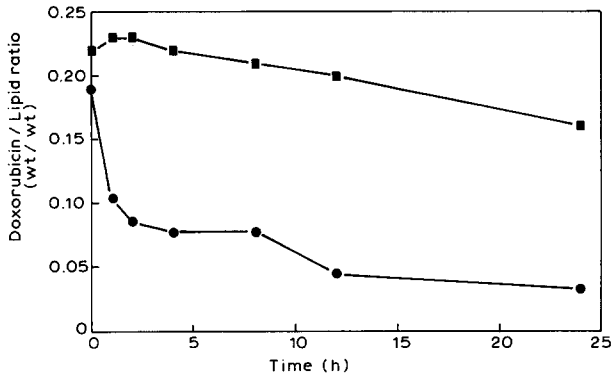

Fig. 6. Drug-to-lipid ratio determined in the plasma of mice treated with 0.12 μm DPPC/chol (solid squares) or 0.12 μm egg PC/chol (solid circles) liposomal doxorubicin.

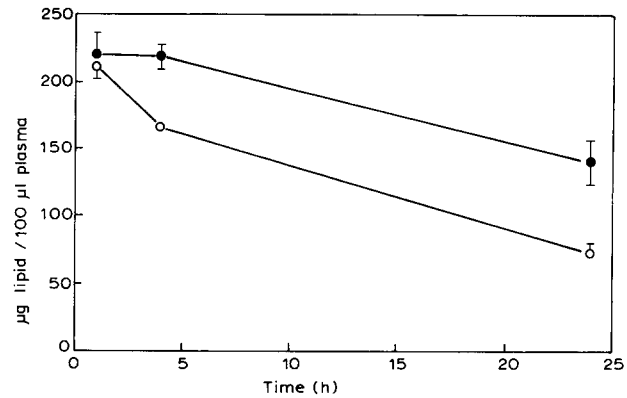

Fig. 7. Influence of liposomal doxorubicin pretreatment on the clearance of empty 0.12 μm DPPC/chol liposomes administered at a lipid dose of 100 mg/kg. One day prior to injection of DPPC/chol empty liposomes the mice were given (i.v.) empty liposomes (10 mg lipid/kg) or liposomal doxorubicin (2 mg doxorubicin/kg; 10 mg lipid/kg). Subsequently the 100 mg/kg dose of liposomes was administered, and at the indicated time points blood was collected. Plasma lipid levels were determined as indicated in Fig. 1. Animals pretreated with liposomal doxorubicin are indicated by the solid circles. Control animals are represented by the open circles. Each point represents the mean of four animals and the error bars indicate the standard deviation.

indicated by the drug-to-lipid radio profiles (Fig. 6), these results show that almost 50% of the entrapped doxorubicin is released from the egg PC/chol liposomes within 1 h following i.v. administration. It is interesting to note that the rate of drug dissociation obtained after one hour (2.08 μg doxorubicin per mg lipid per h) is similar to that obtained for doxorubicin encapsulated in 0.12 μm diameter DPPC/chol liposomes (2.91 μg doxorubicin per mg lipid per h).

The data presented to this point clearly indicate that the presence of entrapped doxorubicin decreases the elimination of the liposomal carrier system from the circulation. This effect cannot be induced by pre-treating animals with free doxorubicin. Administration of free drug (20 mg/kg) 4 or 24 h prior to injection of empty liposomes did not alter the extent of liposome clearance (data not shown). These data support the contention that the decreased elimination of liposomes results specifically from the presence of entrapped drug. It is therefore of interest to determine whether pretreating animals with a lower dose of liposomal doxorubicin can alter the clearance rate of subsequently administered empty liposomes. As illustrated in Table I, 24 h after administration of lipid doses of 10 mg/kg and 100 mg/kg; the percentage of the injected liposome dose in the circulation is 0.32 and 21.67, respectively. For both lipid doses, there is a 2–3-fold increase in the level of liposomal lipid recovered in the plasma when the vesicles contain entrapped doxorubicin. More importantly, pretreating animals with the lower dose of liposomal doxorubicin (2 mg/kg doxorubicin; 10 mg/kg lipid) one day prior to injection of labelled empty liposomes (100 mg/kg) results in a dramatic reduction in plasma clearance (Fig. 7). In particular, the animals which were pretreated with liposomal doxorubicin show no change in the circulating level of liposomal lipid (96% of the injected dose) at 1 h and 4 h after i.v. administration, and at 24 h, 62% of the injected lipid dose remains in circulation of these pretreated animals.

TABLE I

Influence of lipid dose on the recovery of liposomes and liposomal doxorubicin in the plasma of mice 24 h after injection

Liposomes were composed of DPPC/chol and sized through the 0.1 μm filters. Assays for lipid and doxorubicin are as described in the legends of Figs. 1 and 2.

Dose (mg/kg)		% Recovery at 24 h	
lipid	drug	lipid	drug
100	–	21.67	–
10	–	0.32	–
100	21	54.34	45.54
10	2	0.60	0.60
–	20	–	0.02

Discussion

This report shows that small (100 nm) liposomes composed of phosphatidylcholine and cholesterol display prolonged circulation times when administered at appropriate dose levels, and that entrapped doxorubicin markedly reduces the clearance rates of the liposomal carrier system. Here we discuss the considerable biological significance for liposomal delivery systems that are implicit in these results.

The toxicity and therapeutic activity of drugs are known to be dependent on the concentration and/or the lifetime of the agent at the site of potential toxicity or the target site. In this regard many investigators have examined the pharmacokinetic behaviour of doxorubicin [18,19] and of liposomal systems [8] to obtain insight on mechanism of action. However, previous studies on

the elimination of liposomal doxorubicin from the blood [4,5,20,21,22] have focused primarily on the circulating level of doxorubicin and not on the liposome dose required to achieve these circulating drug levels. For example, liposomal doxorubicin (drug-to-lipid ratio of 0.2, w/w) displays antitumour activity equivalent to the free drug when administered at a dose of 20 mg/kg doxorubicin [4]. This doxorubicin dose corresponds to a lipid dose of 100 mg/kg which is high enough to saturate the RES and reduce liposomal clearance rates [8]. This effect would be expected to be even more marked for other liposomal doxorubicin preparations which exhibit drug-to-lipid ratios 3–10-fold lower [4]. A comparable dose of doxorubicin (20 mg/kg) in other formulations would be delivered with lipid doses ranging from 300 mg lipid/kg to well over 1000 mg lipid/kg. Since lipid dose dramatically influences the blood clearance kinetics of liposomes, therapeutic and toxicological studies of liposomal drug formulations must consider the amount of lipid required to achieve a specific drug dose.

The effect of encapsulated drug on the circulation lifetime of liposomal delivery systems has not been observed previously. Doxorubicin is a potent cytotoxic agent [23] and administration of this drug encapsulated in liposomes could result in a direct toxicity to the cells responsible for the clearance of the liposome carrier. Cells of the reticuloendothelial system as well as other phagocytic cell types accumulate liposomal systems [24–26] and subsequent release of the entrapped drug within the cell could result in impaired phagocytic ability or cell death. The data presented in Fig. 7 is consistant with this model of RES toxicity. Pre-treating animals with a low dose of liposomal doxorubicin, which is cleared rapidly from the circulation, effectively blocked the clearance of subsequently administered liposomes. We are currently determining the nature and extent of doxorubicin mediated cytotoxicity on phagocytic cell populations which may accumulate the liposomally entrapped drug.

With regards to the mechanism(s) of biological activity of liposomal doxorubicin, these results do provide new insight. The acute toxicity of these formulations may be governed by the extent of drug dissociation from the liposomal carrier in the circulation. For egg PC/chol liposomal doxorubicin the drug-to-lipid ratio profiles (see Fig. 6) indicate that 50% of the drug dissociates from the carrier within 1 h following i.v. administration. As would be predicted from these results, the LD_{50} dose of doxorubicin entrapped in these liposomes is approximately 2-fold higher than the LD_{50} dose of free drug [4]. The drug-to-lipid ratio profiles also indicate that saturated liposomes (DSPC/chol or DPPC/chol) with entrapped doxorubicin display the greatest in vivo stability. These observations are consistant with previous investigations [7] and with the dramatic reduction in acute toxicity of doxorubicin entrapped in DSPC/chol liposomes [4].

What is remarkable about the liposomal doxorubicin preparations formulated with the saturated lipids concerns the maintenance of biological activity. As indicated elsewhere [4,20], DSPC/chol liposomes with encapsulated doxorubicin display antitumour activity similar to that of the free drug. Further, the increased circulation half-life exhibited for liposome systems with entrapped doxorubicin was most pronounced for vesicles which displayed the greatest in vivo stability. Regardless of the specific nature of the cytotoxic effects which result in this increase in circulation time, these drug mediated changes are detected rapidly (within 4 h) following i.v. administration (see Fig 1), and occur with little or no measurable release of doxorubicin from vesicles within the blood compartment. It is not unreasonable, therefore, to suggest that this biological activity is due to intracellular processing of the saturated liposomes containing doxorubicin. Storm et al. [20], utilizing a liver tumour model, had postulated a similar mechanism for antitumour activity of saturated liposomal doxorubicin systems.

With reference to other tumour models, the increased circulation time exhibited by liposomal doxorubicin systems may result in increased accumulation of the liposomal carrier and associated drug at the sites of tumour growth. It has been demonstrated previously that small vesicles composed of cholesterol and saturated phospholipid species can localize at sites of tumour growth [27]. More recently, Gabizon and Papahadjopoulous [28] have developed and characterized liposomal systems which displays extended circulation times. These liposomal systems also appear to accumulate at sites of tumour growth. The results presented here and elsewhere [4] suggest that drug mediated biological effects can be measured following i.v. administration of stable, long-lived liposomes with entrapped doxorubicin. To date, however, there are no clear indications suggesting that extended circulation times will be of any therapeutic advantage.

In summary, we have demonstrated that the clearance of liposomal doxorubicin from the circulation is governed by the physical characteristics of the liposomes, the cytotoxic activity of the entrapped drug as well as the dose of lipid administered. Specifically, for liposomes composed of distearyol PC/cholesterol administered (i.v.) at a dose of 100 mg/kg 25% of the injected lipid dose is retained in the circulation at 24 h. This value increases to almost 80% when the liposomes contain doxorubicin.

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