Cancer Chemother Pharmacol (1990) 27: 13–19

Cancer Chemotherapy and Pharmacology

Studies on the myelosuppressive activity of doxorubicin entrapped in liposomes

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Received 10 November 1989/Accepted 17 April 1990

Summary. The myelosuppressive activity of doxorubicin encapsulated in liposomes of differing lipid composition and size was quantified in mice by measurement of changes in spleen weight, peripheral white blood cells (WBC), and bone marrow nucleated cells. Following i.v. administration of free doxorubicin at a dose of 20 mg/kg, a 90% reduction in marrow cellularity was observed on day 3. The marrow nucleated cell count was similar to control values by day 7. Administration of an equivalent dose of doxorubicin that was encapsulated in large (diameter, ~1.0 μm) egg phosphatidylcholine/cholesterol (EPC/Chol)(molar ratio, 55:45) liposomes induced an 80% reduction in bone marrow cellularity that lasted for periods of >7 days. Similar results were obtained following administration of large (1.0 μm) liposomal doxorubicin systems formulated with distearoylphosphatidylcholine/cholesterol (DSPC/Chol) (molar ratio 55:45). In contrast, liposomal doxorubicin prepared using small (diameter, ~0.1 μm) DSPC/Chol liposomes induced only a 40% reduction (day 3) in bone marrow cellularity, which returned to control values by day 7. Other indicators of doxorubicin-mediated myelosuppressive activity (spleen weight loss and peripheral leukopenia) correlated well with changes observed in marow cellularity. An exception to this, however, was observed in animals treated with small (0.1-μm) DSPC/Chol liposomal doxorubicin, which displayed peripheral leukopenia for periods of >14 days. This extended leukopenia was not observed following administration of small (0.1-μm) EPC/Chol liposomal doxorubicin. Marrow-associated liposomal lipid and doxorubicin were quantified to determine if the extent of doxorubicin-mediated myeloid toxicity could be correlated to changes in biodistribution of the entrapped drug. It was demonstrated that 10–20 times more doxorubicin is delivered to the bone marrow when the drug is given encapsulated in large liposomes than when it is associated with small liposomes. These data are useful in defining characteristics of liposomal preparations that modulate the myelosuppressive behaviour of entrapped antineoplastic agents.

Introduction

Three different formulations of liposomal doxorubicin are currently being tested in human phase I clinical trials [4, 10, 21, 30, 31]. These liposomal carrier systems were selected on the basis of several preclinical studies, which clearly demonstrated that the therapy limiting cardiotoxicity of free doxorubicin could be reduced when the drug was given in a liposomal form [2, 6, 7, 11, 24]. The reduction in cardiotoxicity has been correlated to changes in the biodistribution of entrapped doxorubicin, by which 2–30 times less doxorubicin is associated with cardiac tissue of animals treated with encapsulated drug [7, 19]. The extent to which drug levels in cardiac tissue are reduced is governed primarily by the lipid composition of the vesicle carrier [19]. This is consistent with the results of previous work, which has shown that lipid composition influences the stability of liposomes in serum and, hence, the degree to which an entrapped agent is released from liposomes following i.v. administration [27]. The antitumour activity of liposomal doxorubicin preparations, on the other hand, appears to be more sensitive to liposome size [8, 19]. Doxorubicin entrapped in small (~1.0 μm) vesicles is significantly more efficacious than an equivalent dose of the drug entrapped in large (~1.0 μm) vesicles [19]. The increased therapeutic activity may be associated with the extended blood-circulation time exhibited by small liposomes [13, 28] and/or the ability of these vesicles to accumulate at sites of tumour growth [22].

The studies summarized above provide a pharmacodynamic basis for selection of liposomal formulations that exhibit reduced toxicity and enhanced therapeutic activity. In this regard, preliminary reports of the phase I clinical

Abbreviations used: MLV, multilamellar vesicle; LuV, large unilamellar vesicle; SUV, small unilamellar vesicle; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Chol, cholesterol; RES, reticuloendothelial system; QELS, quasielastic light scattering; FACS, fluorescence-activated cell sorting; WBC, white blood cell; HBSS, HEPES buffered saline solution

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trials of liposomal doxorubicin indicate that myelosuppression is likely to be the acute dose-limiting toxicity [10, 31]. Previous preclinical studies have compared the myelosuppressive and/or immunotoxic properties of free doxorubicin vs doxorubicin entrapped in liposomes [9, 23]. However, an evaluation as to how the physical characteristics of liposomal systems influence the myelosuppressive activity of the entrapped drug has not been performed. This is surprising, considering that it has been reported that small liposomes can localize in the marrow compartment following i.v. administration [28], implying that delivery of entrapped agents to this site could be influenced by changes in the size of the vesicle carrier. The present study investigates the way in which changes in vesicle size and lipid composition influence doxorubicin's delivery to and activity on the bone marrow. The data demonstrate that the extent and duration of myelosuppression is strongly correlated with vesicle-mediated changes in drug biodistribution.

Materials and methods

Animals. Female DBA/2J mice, 6–8 weeks old, were obtained from Jackson Animal Laboratories (California, USA). Groups of four mice per experimental point were given the specified treatment as a single i.v. dose via a lateral tail vein. The dose, given in a volume of 200µl, was based on mean body weight. Marrow samples were obtained from a single hind leg of a mouse. The tibia and femur were isolated and flushed with 10 ml Hanks' buffered saline solution (indicator-free). Subsequently, the cells were pelleted at 200 *g* and resuspended in 1 ml 2% acetic acid to lyse the red blood cells. Marrow cell and peripheral WBC counts were made using standard haemacytometric techniques. Blood samples were collected via heart puncture and placed into ethylenediaminetetraacetate (EDTA)-containing microtainer tubes (Becton Dickinson). Subsequently, the samples were subjected to 10-fold dilution in 2% acetic acid to lyse the red blood cells.

Liposome preparation. Egg phosphatidylcholine (EPC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidiylcholine (DSPC) were purchased from Avanti Polar Lipids (Alabama, USA). Cholesterol (Chol) was obtained from Sigma Chemicals (St Louis, USA), and all other chemicals were of reagent grade. Lipid mixtures were prepared in a chloroform solution and subsequently dried under a stream of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2 h.

Multilamellar vesicles (MLVs, 100 mg/ml) were formed by hydration of the dried lipid with 300 m*M* citric acid (pH 4.0). The resulting preparation was frozen and thawed 5 times prior to extrusion 10 times through two stacked polycarbonate filters (Nuclepore) of the indicated pore size [12]. When DSPC or DPPC were used, the sample and extrusion apparatus (Lipex Biomembranes, Inc., Vancouver, Canada) were heated to 5° C above the thermotropic phase transition temperature of the phospholipid prior to extrusion [20]. Liposome particle size was determined by quasielastic light scattering (QELS) measurements (using a Nicomp 370 particle sizer operating at a wavelength of 632.8 nm). QELS measurements indicated that liposomes prepared by sizing of MLVs through 1.0-µm filters exhibited mean diameters ranging from 1.1 to 1.7 µm; the mean diameters of MLVs sized through 0.1-µm filters (depending on lipid composition and particular filter lot) ranged from 0.12 to 0.14 µm. These vesicle preparations are henceforth referred to as 1.0-µm or 0.1-µm liposomes.

Doxorubicin encapsulation. Doxorubicin (obtained from Adria Laboratories, Inc., Mississauga, Ontario) was encapsulated as previously described [18, 19]. The liposome suspension (pH 4.0) was adjusted to pH 8.0–8.5 with 0.5 *M* Na_2CO_3. The liposome preparation was heated to 60° C and then transferred to a preheated (60° C) vial of doxorubicin, typically with the addition of enough lipid to achieve a final drug-to-lipid ratio of 0.2:1 (wt/wt). This mixture was incubated with intermittent mixing for 10 min at 60° C. Doxorubicin concentration, lipid concentration and encapsulation efficiency were determined as previously described [19]. This procedure resulted in entrapment efficiencies of >98%. Solutions for injection were prepared with sterile physiological saline such that the specified dose could be delivered in a volume of 200 µl.

For this study, equivalent drug doses were used rather than doses that were equi-toxic or therapeutically equivalent, to ensure that comparisons between liposomal formulations could be made following administration of similar lipid doses (100 mg/kg). As indicated elsewhere [19], the LD_{50} (lethal dose for 50% of a group) of free doxorubicin in DBA/2J mice is 25 mg/kg, whereas the EPC/Chol and DSPC/Chol liposomal doxorubicin systems exhibit an LD_{50} of 40 and 80 mg/kg, respectively. At equivalent doses of doxorubicin, the antitumour activity (L1210 murine tumour model) of the small (~0.1 µm) liposomal doxorubicin systems is significantly greater than that of the large (~1.0 µm) preparations [19]. Furthermore, the LD_{50} decreases as vesicle size decreases; for EPC/Chol liposomes, LD_{50} values of 60 and 45 mg/kg were obtained in CDl mice for preparations exhibiting mean diameters of 1,400 and 106 nm, respectively.

Quantitation of liposomal lipid and doxorubicin. Liposomal lipid was measured using the lipid marker [^{3}H]-cholesteryl hexadecyl ether (Dupont-NEN). Previous studies have demonstrated that this lipid label is non-exchangeable and non-metabolizable [14, 25], and liposomal clearance studies from our laboratory [3] have confirmed its non-exchangeability. For scintillation counting, bone marrow samples (450 µl) in HEPES buffered saline solution (HBSS) or 2% acetic acid were mixed with 5 ml Pico-Fluor 40 scintillation cocktail (Packard, Canada) and then counted using a Beckman LS3801 scintillation counter.

Doxorubicin accumulation was determined using a fluorescent assay procedure. Bone marrow samples (450 µl) were diluted to 1 ml in physiological saline adjusted to pH 4.0 with HCl. The sample was extracted with 2 ml chloroform/isopropyl-alcohol (1:1 v/v). Following vigorous mixing and brief centrifugation, the organic phase was collected. Fluorescence of the organic phase was determined (excitation wavelength, 500 nm; emission wavelength, 550 nm) with a Shimadzu RF-540 spectrofluorophotometer. A standard doxorubicin curve was prepared using a similar extraction procedure in the presence and absence of cells. Values for lipid and doxorubicin are expressed as ng/10^6 bone marrow nucleated cells or total nanograms associated with samples isolated from a single hind leg. HPLC analysis of various marrow samples indicated that >90% of the fluorescence detected in the doxorubicin assay was attributable to the native drug.

FACS analysis. Bone marrow cells were isolated 24 h after treatment with the specified preparation. These cells were flushed from the bone with cold, sterile HBSS. Subsequently the cells were pelleted and erythrocytes were lysed using ice-cold ammonium chloride buffer. The cells were washed twice with cold HBSS and then resuspended in 1–2 ml HBSS with 5% fetal bovine serum. The fluorescence of the cells was further analyzed using a FACS 440 system (Becton-Dickinson), with the laser operating at 488 nm (0.3 W). Doxorubicin fluorescence was detected with the same band-pass filters used for fluorescein (530 nm). Viable cells were separated from non-viable cells on the FACS using pyridinium iodide as a fluorescent dye-exclusion marker. Identifications of cell subpopulations were based on previous studies using cell-specific fluorescently labeled antibodies to tag specific cell populations. These identifications were not confirmed in the present studies.

Statistical analysis. A two-tailed Student's *t*-test for unpaired data was performed to compare groups treated with liposomal systems of different size and/or different lipid composition.

Results

Bone marrow toxicities of myelosuppressive drugs in murine models have been shown to be similar to those observed in humans [17]. In this regard, changes in bone marrow cellularity appear to provide the most direct measure of drug-induced myelosuppression in murine

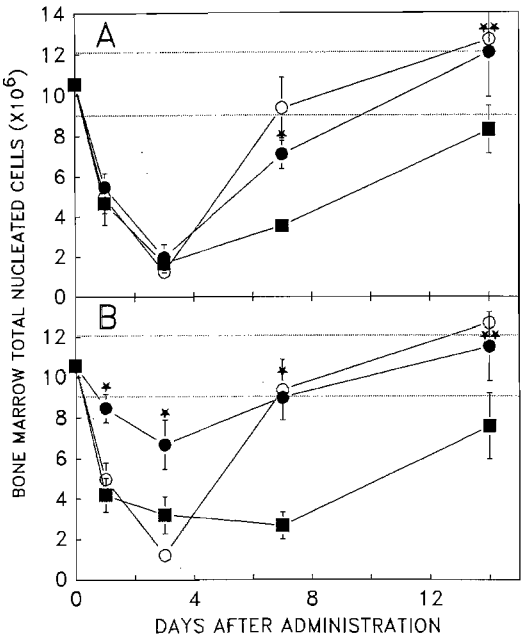

Fig. 1 A, B. Bone marrow nucleated cell counts determined in mice following administration of free doxorubicin (*open circles*), **A** EPC/Chol liposomal doxorubicin, and **B** DSPC/Chol liposomal doxorubicin. The liposomes were prepared through 0.1-μm pore filters (*filled circles*) or 1.0-μm pore filters (*filled squares*). Points represent the mean obtained from at least 4 animals and the error bars indicate the standard deviation. The *dotted lines* represent the range of values obtained for control animals ($n = 8$). Statistical comparisons between groups treated with 0.1 μm vs 1.0 μm liposomes are indicated: $*P < 0.01$; $**P < 0.05$

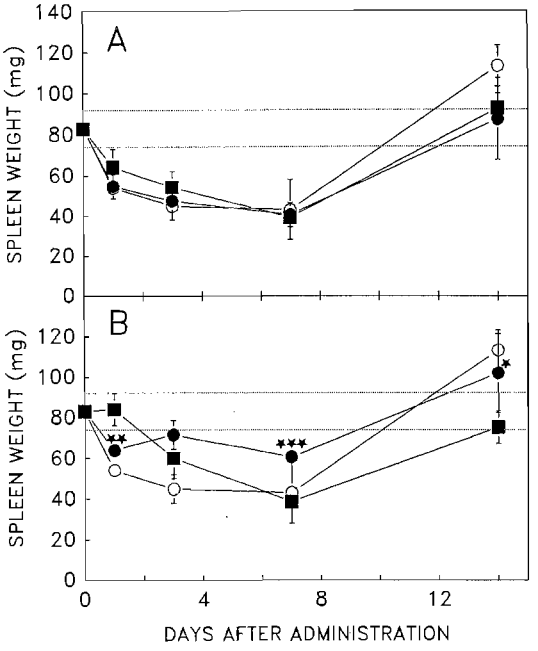

Fig. 2 A, B. Spleen weights of animals treated with free doxorubicin (*open circles*), **A** EPC/Chol liposomal doxorubicin, or **B** DSPC/Chol liposomal doxorubicin. The liposomes were prepared by extrusion through 0.1-μm pore filters (*filled circles*) or 1.0-μm pore filters (*filled squares*). Points represent the mean of at least 4 animals, with error bars indicating the standard deviation for each group. The *dotted lines* represent the range of spleen weights obtained for control animals ($n = 18$). Statistical comparisons between groups treated with 0.1 μm vs 1.0 μm liposomes are indicated: $*P < 0.02$; $**P < 0.01$; $***P < 0.001$

models. This is illustrated in Fig. 1 for animals given free doxorubicin i.v. at a dose of 20 mg/kg (open symbols). This doxorubicin dose resulted in a pronounced reduction in bone marrow cells. The nadir was obtained by day 3, when a 90% reduction ($P < 0.001$ relative to control values) in total nucleated cells was observed; by day 7 the counts were similar to control values, and some hypercellularity was indicated by day 14. Marrow cellularity was well within the control range by day 21 (data not shown).

Figure 1 also indicates the influence of liposomally entrapped doxorubicin on marrow cellularity. Liposomes composed of either EPC/Chol (55:45, Fig. 1A) or DSPC/Chol (55:45, Fig. 1B), exhibiting mean diameters of approximately 1.0 μm (filled squares) or 0.1 μm (filled circles), were loaded with doxorubicin as indicated in Materials and methods to achieve a final drug-to-lipid ratio of 0.2:1 (wt/wt). These liposomal doxorubicin systems were injected i.v. at a dose equivalent to that of the free drug (20 mg/kg). As shown in Fig. 1A, animals treated with 1.0 μm or 0.1 μm EPC/Chol liposomal doxorubicin displayed nucleated cell counts on days 1 and 3 that were similar to those observed following administration of free drug. The recovery time for this cell population was more rapid in animals treated with the small doxorubicin-containing vesicles vs the large systems. Animals treated with 1.0 μm EPC/Chol liposomal doxorubicin continued to display a 75% reduction in marrow nucleated cells on day 7, with cell counts being slightly below control values on day 14 after administration. Liposomal doxorubicin-induced suppression of marrow nucleated cells was also observed following administration of the DSPC/Chol systems (Fig. 1B). On days 1, 3 and 7 after injection of the 1.0-μm DSPC/Chol liposomal doxorubicin, a 60%–75% reduction in marrow nucleated cells was seen. The cell counts on day 14 were significantly ($P < 0.05$) below control values. In contrast, administration of 0.1-μm DSPC/Chol liposomal doxorubicin induced only a 40% reduction in marrow nucleated cells on day 3. This was significantly less ($P < 0.01$) than the 75% and 90% values observed following injection of 1.0-μm DSPC/Chol liposomal doxorubicin and free doxorubicin, respectively. Furthermore, animals treated with the 0.1-μm DSPC/Chol systems showed marrow cell counts similar to control values by day 7.

In addition to measurements of bone marrow cellularity, a preliminary (stage 1) evaluation of myeloid-associated toxicities often includes routine haematology and characterization of lymphoid organs such as the spleen [26]. Doxorubicin-mediated changes in spleen weight are shown in Fig. 2. The extent and duration of spleen weight loss observed for the free drug was similar to that seen when the drug was entrapped in EPC/Chol vesicles, regardless of the vesicle size used (Fig. 2A). When DSPC/Chol, liposomal doxorubicin was injected, there were differences in the influence of the entrapped drug on this lymphoid organ (Fig. 2B). First, following administration of 1.0-μm DSPC/Chol liposomal doxorubicin, there was a delay in the onset of organ weight loss. The nadir, obtained on day 7, was similar to that observed with the free drug. Second, the 0.1-μm DSPC/Chol liposomal doxo-

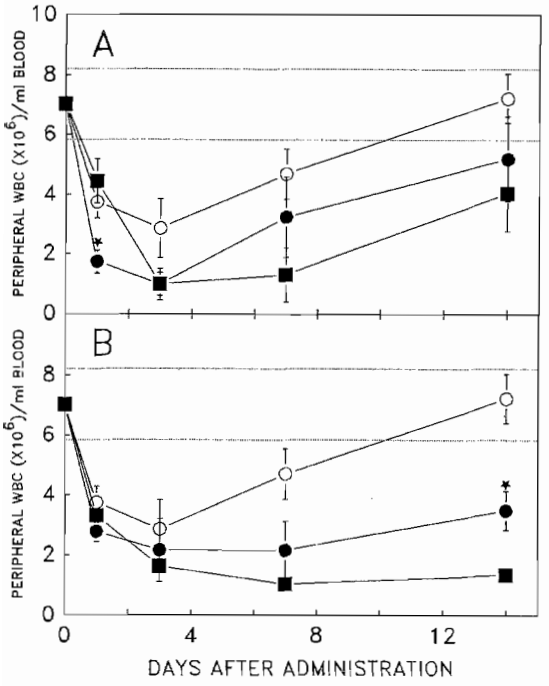

Fig. 3 A, B. Peripheral white blood cell (*WBC*) counts determined in mice following administration of free doxorubicin (*open circles*), **A** EPC/Chol liposomal doxorubicin, and **B** DSPC/Chol liposomal doxorubicin. The liposomes were prepared as described in Materials and methods, with vesicles being sized through 0.1-μm pore filters (*filled circles*) or 1.0-μm pore filters (*filled squares*). Points represent the mean obtained from at least 4 animals and the error bars indicate the standard deviation. The *dotted lines* represent the range of values obtained for control animals ($n = 8$). Statistical comparisons between groups treated with 0.1 vs 1.0-μm liposomes are indicated: $^*P < 0.01$. Values for groups treated with DSPC/Chol liposomal doxorubicin, regardless of size, were significantly different from those obtained with free drug on day 7 ($P < 0.01$) and day 14 ($P < 0.005$)

rubicin system was significantly ($P < 0.01$) less effective at inducing spleen weight loss than was either free drug or doxorubicin encapsulated in 1.0-μm DSPC/Chol liposomes. A 23% reduction in spleen weight was observed on day 1; however, this did not progress further, and by day 14 values had returned to levels similar to those seen in controls.

The peripheral WBC counts should reflect changes occurring in bone marrow cellularity. This is demonstrated in Fig. 3 for the free drug (open symbols). Peripheral WBCs reached a nadir (40% of control counts) on day 3 and recovered to control values by day 14, similar to events in the marrow cell population. The results shown in Fig. 3 also indicate that the liposomal form of the drug, regardless of lipid composition or size, was more effective at inducing leukopenia in mice than was the free drug. The 1.0-μm liposomal doxorubicin systems induced the greatest decrease (80% at the nadir) in peripheral WBCs. Following administration of these preparations, the peripheral WBC count was depressed for periods of at least 14 days. These animals exhibited cell counts similar to those of controls on day 21 (data not shown). The 0.1-μm liposomal doxorubicin formulations appeared to induce a rapid (day 1) 60%–75% reduction in peripheral WBCs. By day 14, animals treated with the small EPC/Chol liposomal systems

Table 1. Assessment of bone marrow nucleated cells by FACS[a]

	Percentage of cells counted			
	Control	0.1 μm	1.0 μm	Free
Total viable cells	86.4	87.1	80.8	86.8
Non viable cells[b]	12.5	13.6	23.1	14.7
Viable cells (subpopulations):				
Population 1 (lymphoid cells)[c]	32.8	36.5	39.2	35.6
Population 2 (blast cells)	21.8	18.2	18.5	11.5
Population 3 (myeloid cells)	43.9	44.0	41.1	52.1
Fluorescent cells[d]				
Total cells		2.3	7.9	2.3
Viable cells		0.1	0.3	0.1

[a] Bone marrow cells were isolated as indicated in Materials and methods at 24 h after administration of the indicated preparation. Liposomes were composed of DPPC/Chol (55 : 45) and were given at a drug dose of 20 mg/kg. Data are representative of 2 experiments, and values indicate the percentage of cells counted. A minimum of 20,000 cells were counted twice for each sample

[b] Viability was assessed using the fluorescent dye-exclusion marker pyridinium iodide

[c] FLS and PLS analyses indicated three cell subpopulations. These were defined on the basis of previous studies using cell specific fluorescently labeled antibodies to tag each cell population

[d] Fluorescence due to the presence of doxorubicin

showed WBC counts similar to those obtained following administration of free drug. It is surprising that the WBC count of animals treated with the small DSPC/Chol systems were significantly ($P < 0.01$) below control counts for periods of up to 14 days following administration. This drug-induced leukopenia did not reflect events measured in the bone marrow (Fig. 1 B) or spleen (Fig. 2B) but may indicate toxicities at other lymphopoietic organs such as the thymus.

In determinations of bone marrow counts, it was noted for animals treated with the large (1.0-μm) DSPC/Chol liposomal doxorubicin systems that many of the cells counted by light microscopy appeared to be red, suggesting the presence of cell-associated drug. Since doxorubicin is a fluorescent compound, the presence of cell-associated drug could be analysed using fluorescence-activated cell-sorting techniques (FACS). Mice were treated with free drug or with 0.1 or 1.0-μm DPPC/Chol liposomal doxorubicin. Liposomal doxorubicin prepared with DPPC/Chol liposomes exhibited plasma clearance profiles [3] and LD_{50} values (Bally, unpublished observation) comparable with those obtained using DSPC/Chol liposomal doxorubicin. Both of these saturated liposomal formulations are stable following i.v. administration (as indicated by in vivo plasma-release studies; see [3]), with DPPC/Chol liposomal doxorubicin exhibiting in vivo drug-dissociation rates slightly faster than those of DSPC/Chol liposomal doxorubicin. At 1 day after i.v. administration of drug, marrow nucleated cells were isolated (see Materials and methods) and viable cells were separated by FACS using pyridinium iodide as a fluorescent dye-exclusion marker. Results from a representative experiment are shown in Table 1.

The combination of FLS (forward light scatter) and PLS (perpendicular light scatter) revealed three distinct

Table 2. Influence of vesicle size on the accumulation of liposomal doxorubicin in the marrow compartment

	Total marrow associated[a]	
	Lipid (μg)	Drug (μg)
1.0 μm empty liposomes[b]	17.9±2.2	–
1.0 μm liposomal doxorubicin	23.2±6.2	1.5±0.2
0.1 μm empty liposomes	4.1±0.5*	–
0.1 μm liposomal doxorubicin	8.8±3.0**	0.4±0.1

[a] Bone marrow was isolated at 24 h as indicated in Materials and methods using 5 ml HBSS. The cells and media were subsequently assayed for liposomal lipid and drug. Values represent the mean of at least four animals (± SD) and account for the delivery of drug to a single hind leg

[b] Composed of DPPC/Chol (55:45), with a drug-to-lipid ratio of 0.2 (wt/wt), and injected at a dose of 20 mg/kg

* Significantly different from values for 1.0 μm empty liposomes (P <0.001)

** Significantly different from values for 0.1 μm empty liposomes (P <0.05).

cell subpopulations that were tentatively defined (based on previous studies using cell-specific labeling techniques) as lymphoid cells (population 1), blast cells (population 2) and myeloid cells (population 3). Three points can be made from the data in Table 1. First, there were greater proportion of non-viable cells found in marrow samples of animals treated with 1.0-μm liposomal doxorubicin. Second, analysis of the cell subpopulations indicates that the free drug caused a relative decrease in population 2 (blast cells). This is consistent with results of previous investigations showing that blast cells accumulate the drug to a greater extent than do other marrow cell types [29]. This effect was not observed for either the large or the small liposomal doxorubicin preparations. Finally, of the cells that were fluorescent (~8% for animals treated with 1.0-μm liposomal doxorubicin), nearly all were non-viable as indicated by pyridinium iodide dye exclusion.

The present data suggest that doxorubicin can accumulate to a greater extent in bone marrow nucleated cells when the drug is encapsulated in 1.0-μm vesicles. Previous studies, however, have suggested that small vesicles (<0.1 μm) have access to and can accumulate in the bone marrow [28]. To determine the influence of vesicle size on the accumulation of liposomal doxorubicin in the marrow compartment, both liposomal lipid and doxorubicin were quantified. These data are shown in Fig. 4 and Table 2. As would be expected from the flow cytometry studies, 4 times more drug (and lipid) was observed in the marrow compartment (cells plus media used to isolate cells) at 24 h in animals treated with 1.0-μm vesicles vs 0.1-μm vesicles (Table 2).

These values were not corrected for plasma volume in the bone marrow. As indicated elsewhere [3], at 24 h following administration of the small DPPC/Chol vesicles, 56% of the injected lipid dose was retained in the circulation, as opposed to 3% of the injected lipid dose for large vesicles. Therefore, plasma levels of lipid and drug would contribute significantly to the marrow levels only in animals given 0.1-μm liposomal doxorubicin. This is supported by the observed 2-fold increase in liposomal lipid

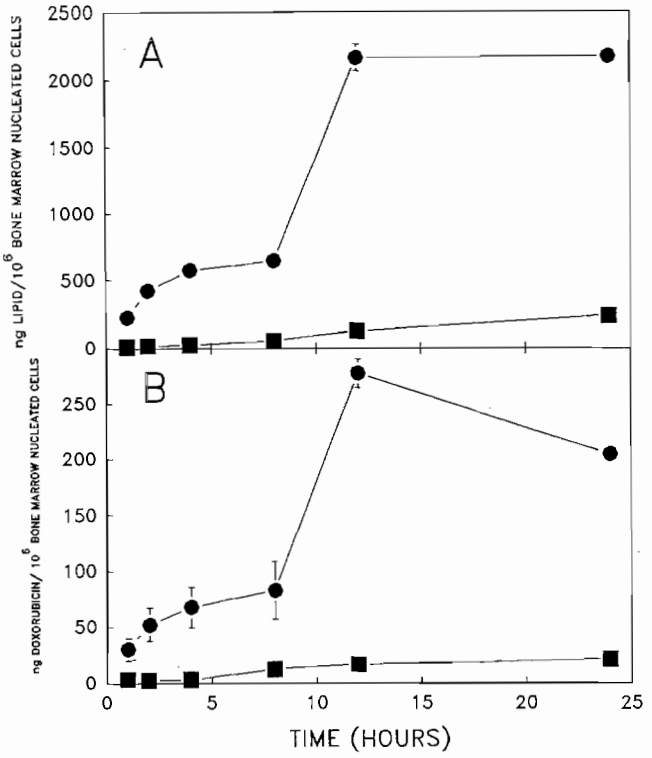

Fig. 4 A, B. The influence of vesicle size on the accumulation of **A** *lipid (liposomes) and* **B** doxorubicin in bone marrow nucleated cells. DPPC/Chol liposomal doxorubicin prepared with vesicles sized through 1.0-μm (*filled circles*) or 0.1-μm (*filled squares*) filters was injected at a dose of 20 mg/kg doxorubicin or 100 mg lipid/kg [the drug-to-lipid ratio was 0.2:1 (wt/wt)]. Doxorubicin and liposomal lipid were assayed as indicated in Materials and methods. Points represent the mean of at least 4 animals and the error bars indicate the standard deviation

found in marrow compartments of animals receiving 0.1-μm liposomal doxorubicin vs identically prepared 0.1-μm empty liposomes and is consistent with the increased blood residence times observed for liposomes containing entrapped drug [3]. Furthermore, in animals treated with the 0.1-μm liposomal preparation, 80% of the liposomal lipid found in the marrow compartment was not cell-associated. In contrast, almost 70% of the liposomal lipid was cell-associated in animals treated with 1.0-μm liposomal doxorubicin. These differences are reflected by the data in Fig. 4, which show the time course for the accumulation of liposomal lipid and drug in marrow cells. In all, 10–20 times more liposomal lipid (Fig. 4A) and doxorubicin (Fig. 4B) were associated with marrow nucleated cells when 1.0-μm DPPC/Chol liposomal doxorubicin was given. The increase in cell-associated liposomal doxorubicin measured at 12 h coincided with a 40% reduction in bone marrow nucleated cells observed simultaneously. A 60% reduction in marrow nucleated cells was observed at 24 h, consistent with observations shown in Fig. 1 for 1.0-μm DSPC/Chol liposomal doxorubicin.

The data in table 2 also indicate that empty liposomes, as well as doxorubicin-loaded liposomes, accumulated in the marrow compartment. As shown in Fig. 5A, the time course for uptake of empty vs loaded 1.0-μm liposomes was identical over the first 8 h. Within 4 h after administration of the liposomes, approximately 500 ng lipid/10^6 mar-

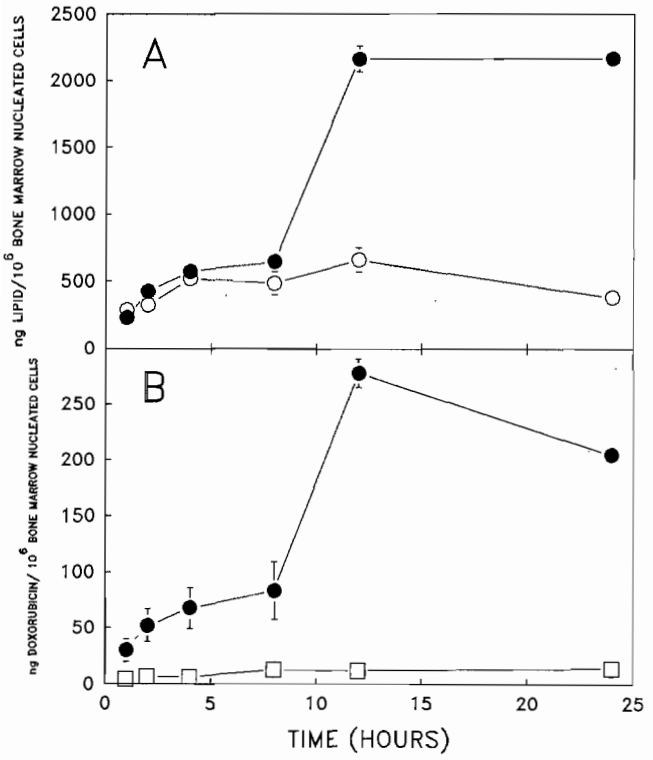

Fig. 5 A, B. Quantification of bone marrow nucleated cell-associated **A** lipid (liposomes) and **B** doxorubicin after administration of free doxorubicin (20 mg/kg; *open squares*), empty 1.0-μm DPPC/Chol liposomes (100 mg lipid/kg; *open circles*) and 1.0-μm DPPC/Chol liposomal doxorubicin [20 mg/kg doxorubicin given such that the drug-to-lipid ratio was 0.2 : 1 (wt/wt); *filled circles*]. Each point represents the mean of at least 4 animals and the error bars indicate the standard deviation

row nucleated cells became cell-associated. This corresponds to localization of approximately 0.28% of the injected lipid dose in the marrow isolated from a single leg. Figure 5B indicates the difference in accumulation of doxorubicin in the marrow cells following administration of free drug vs 1.0-μm DPPC/Chol liposomal doxorubicin. A 10-fold increase in the delivery of the drug to the marrow cells was observed when the drug was entrapped in 1.0-μm liposomes.

Discussion

In this investigation the myelosuppressive activity of doxorubicin is correlated to the accumulation of liposomes and entrapped drug in the marrow compartment. The data clearly demonstrate that large (1.0-μm) liposomal systems can deliver greater quantities of entrapped drug to the marrow compartment. The increased accumulation of drug in the marrow is reflected by a pronounced and extended suppression of marrow cellularity. The myelosuppressive activity of liposomally encapsulated antineoplastic agents can therefore be influenced by the physical properties of the vesicles used.

Although 1.0-μm liposomes appear to associate with marrow cells to a greater extent than do 0.1 μm vesicles, a similar number of particles may be cell-associated for both preparations. Using freeze-fracture protocols to determine the number of particles in a given fracture plane, it can be estimated that an equal number of 0.1 and 1.0-μm vesicles are present when the large systems have a lipid concentration approximately 7 times greater than that of the small vesicles (M. J. Hope, unpublished observation). Assuming that the same number of particles (liposomes) become cell-associated, 7 times more liposomal lipid would be associated with the marrow cells following administration of 1.0-μm liposomes. This is comparable with the differences in marrow uptake obtained in the present study for 0.1 vs 1.0-μm vesicles (Fig. 5).

This study did not attempt to identify the marrow cell population that accumulates liposomal doxorubicin. However, resident bone marrow macrophages [15] are most likely responsible. This is supported by the fact that liposome uptake by marrow cells occurs over 4 h (Fig. 4), a time course similar to that obtained for cultured bone marrow macrophages (R. Nayar, unpublished observation). It is noteworthy that at 12 h following treatment with 1.0-μm liposomal doxorubicin, a 40% reduction in marrow nucleated cell count was observed, which coincides with an apparent 2- to 3-fold increase in the level of marrow cell-associated lipid and drug. This suggests that cell populations other than those that have accumulated liposomal drug are being eliminated from the marrow compartment. Release of the drug from cells that have accumulated liposomal doxorubicin may be responsible for the prolonged decrease in marrow cellularity observed in this study (Fig. 1B). This interpretation is supported by the following observations. The 1.0-μm liposomal doxorubicin systems have been shown to be rapidly cleared from the circulation (see [3]), such that only 2%–3% of the injected doxorubicin dose remains after 24 h. By day 3, no doxorubicin can be detected in the plasma of these animals. In contrast, as indicated by fluorescence microscopy, cells containing doxorubicin are present in marrow samples isolated 7 days after i.v. administration of 1.0-μm DSPC/Chol liposomal doxorubicin (data not shown). It therefore appears that doxorubicin-mediated marrow cell suppression may result primarily from liposomes that had previously been sequestered in the marrow compartment.

Doxorubicin-mediated suppression of marrow cells was generally reflected by changes in spleen weight and peripheral WBC count. An exception to this correlation occurred in animals receiving 0.1-μm DSPC/Chol liposomal doxorubicin; peripheral cell counts (Fig. 3B) indicated a pronounced and extended leukopenia following administration of this preparation, whereas changes in bone marrow cellularity (Fig. 1B) and spleen weight (Fig. 2B) indicated relatively minor and transient suppression. Clearly this represents an area of interest in evaluation of the myelosuppressive activity of liposomally entrapped antineoplastic agents. It is not clear why peripheal WBC counts did not follow changes in marrow cellularity in animals treated with 0.1-μm DSPC/Chol liposomal doxorubicin. This may have resulted from toxicities in other lymphopoietic organs, or it is possible that this liposomal doxorubicin preparation was exerting direct toxicity on circulating WBCs through a specific cell/liposome association. Previous studies have shown that leukocytes can bind and endocytose liposomal systems [5, 16]. The prolonged leukopenia observed in the present study would be

consistent with the extremely long circulation lifetimes of the small DSPC/Chol liposomal doxorubicin systems [3]. Following administration of this preparation, 60% of the injected drug dose remains in the circulation at 24 h, 10% is measured in the circulation on day 3 and detectable levels of doxorubicin have been measured in the plasma 14 days after injection [3].

Myelosuppression is a severe toxicity exhibited by a variety of antineoplastic agents. The present results indicate that this toxicity could be manipulated by encapsulating these drugs in liposomal carriers. For example, doxorubicin encapsulated in small liposomes composed of long-chain, saturated phosphatidylcholine species and cholesterol exhibited reduced effects on bone marrow cellularity (Fig. 1B), minimal cardiotoxicity [19], and therapeutic effects equivalent to those of the free drug [19]. It is not clear whether the prolonged leukopenia observed following administration of small DSPC/Chol liposomal doxorubicin systems will result in immune suppression or compromise the ability of the animal to fight infections. Additional studies examining the myelosuppressive effects of liposomal doxorubicin encapsulated in 0.1-μm DSPC/Chol vesicles and given at equitoxic doses (i.e. LD_{10}) relative to the free drug must be done.

In summary, the present results demonstrate that large liposomal doxorubicin systems deliver more drug to marrow cells than do small liposomes. The difference in biodistribution correlated with drug-induced suppression of marrow cells. The data suggest that the myelosuppressive activity of liposomal anticancer drugs can be regulated through changes in the physical characteristics of the vesicle carrier.

Acknowledgements. This research was supported by the National Cancer Institute of Canada and The Liposome Company, Inc. (Princeton, N. N.). The authors wish to thank V. Dragowska for her assistance with the fluorescence-activated cell sorter and Dr. C. Eaves for her careful review of this manuscript.

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