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Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumor-bearing mice

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Summary. Previously we have demonstrated that the L1210 antitumor activity of liposomal doxorubicin increased significantly as the size of the liposomal carrier was reduced from 1.0 to 0.1 μm. It is demonstrated herein that empty and drug-loaded small (0.1-μm diameter) liposomes accumulate efficiently into the peritoneal cavity of normal and ascitic L1210 tumor-bearing animals following i.v. administration. In normal mice injected with 100 nm DSPC/chol liposomal doxorubicin (drug-to-lipid ratio of 0.2; wt/wt) approximately 2.8 μg drug could be recovered from the peritoneal cavity following peritoneal lavage at 24 h. Although this represents only 0.7% of the injected doxorubicin dose, this level of drug is 2 orders of magnitude greater than that achieved following administration of an equivalent dose of free drug (20 mg/kg). The drug levels achieved within the peritoneal cavity are dependent on the physical characteristics (size, drug-to-lipid ratio and lipid composition) of the liposomes employed. Optimal delivery is obtained employing 100 nm DSPC/chol liposomal doxorubicin, a vesicle system that is known to retain entrapped drug following i.v. administration and exhibits extended circulation lifetimes. Analysis of drug and liposome distribution within the peritoneal cavity of normal mice indicates that as much as 50% of the measured doxorubicin and liposomal lipid is cell-associated. Flow cytometric analysis of the peritoneal cells demonstrated that cell-associated doxorubicin resides almost exclusively within resident peritoneal macrophages. The increased delivery of doxorubicin to the peritoneal cavity of normal mice following i.v. administration of small (0.1-μm) liposomal doxorubicin is correlated with a pronounced (>90%) and prolonged (>14-day) suppression of resident peritoneal cells. Liposomal drug accumulation increased dramatically in animals with an established L1210 ascitic tumor. More than 5% of the injected dose was found in the peritoneal cavity of these animals 24 h after treatment with DSPC/chol liposomal doxorubicin as compared with a value of 0.03% of the injected dose achieved with free drug. It is proposed that accumulation of liposomes into the peritoneal cavity of normal and tumor-bearing mice may serve as a useful model for characterizing factors mediating the transfer of liposomes from the vascular compartment to extravascular sites.

Key words: Liposomes – doxorubucin – extravasation

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Introduction

The mechanism(s) responsible for reduction in the toxicity of liposomal doxorubicin have been established on the basis of altered pharmacokinetic and biodistribution characteristics of the entrapped drug [16, 17, 28, 40]. It is not clear, however, how liposome-mediated changes in the pharmacodynamic behaviour of encapsulated doxorubicin influences therapeutic activity. Many studies have shown that following i.v. administration of liposomes, encapsulated contents accumulate to significant levels in sites of infection [2], inflammation [37, 47] and tumor growth [11, 14, 15, 37, 39, 41]. Recent studies have concentrated on characterizing and optimizing liposomal carriers for delivery of contents to tumors and establishing improved therapeutic activity for such liposomes containing anticancer drugs [11, 14, 15, 37, 45]. It is apparent that drugs and other associated markers accumulate more efficiently in disease sites when entrapped in liposomes that exhibit extended circulation lifetimes [14, 37]. To date, increased circulation longevity has been best achieved by incorporation of specialized lipids such as the ganglioside GM_1[1], with polyethylene glycol-modified phospholipids [23] or, more recently, with a glucuronic acid phospholipid derivative [35]. It has also been shown that entrapped cytotoxic drugs significantly increase the circulation lifetime of an associated liposomal carrier [3].

Tumor localization following i.v. administration of liposomal drugs has been rationalized on the basis that circulating liposomes serve as depots for drug within the

plasma compartment [30]. Release of encapsulated contents from liposomes in the vascular system could increase exposure and, hence, promote accumulation within a disease site. Alternatively, it can be suggested that liposomes optimize delivery of encapsulated contents to other circulating macromolecules such as transferrin or lipoproteins, which could subsequently transfer the agent to an extravascular site. Recently, however, studies have shown that the liposomal carrier itself accumulates within tumors [29]. This observation is surprising, given the stringent biological barriers that exist to prevent leakage of circulating macromolecules (such as liposomes) to extravascular sites [21]. The extravasation of liposomal carriers, however, may be due to unique vascular structures arising within tumors or to tumor-mediated changes in host vascular structure. Certain tumors, for example, are known to contain fenestrated endothelium as well as postcapillary venules, regions where macromolecules can move from the vascular compartment to extravascular regions through openings between vascular endothelial cells [18, 19, 21, 42, 44, 46]. In addition, it is known that certain tumors can release factors that increase vascular permeability [5, 6, 22]. These molecules act by inducing pore formation between endothelial cells [5] as well as by promoting transcytosis through endothelial cells [22].

The studies described herein examine the transport of empty and drug-loaded liposomes from the plasma compartment to the peritoneal cavity of normal and ascites tumor-bearing animals. A similar model has been used elsewhere for characterizing the transport of serum-associated proteins and other circulating macromolecules across vascular endothelium [10, 24, 32]. Blood vessels lining the peritoneal cavity of normal mice are relatively impermeable to circulating macromolecules; therefore, peritoneal influx should represent a more stringent test for determining whether liposomal carriers can access extravascular sites. The data presented herein and reported in preliminary form elsewhere [4] show that small (~ 0.1-μm) liposomes can accumulate in the peritoneal cavity of normal mice following i.v. administration.

Materials and methods

Materials. Egg phosphatidylcholine (egg PC), dipalmitoylphosphatidylcholine (DPPC) and distearylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids (Birmingham, Ala.); cholesterol and other reagent-grade chemicals, from Sigma Chemicals (St. Louis, Mo.); doxorubicin, from Adria Laboratories (Mississauga, Ont., Canada); [^{3}H]-cholesterol hexadecyl ether, from NEN (Du Pont Canada, Mississauga, Ont.); and Pico-Fluor 40 scintillation cocktail, from Canberra Packard Canada (Mississauga, Ont.).

Animals. Female DBA/2J mice, 6–8 weeks old, were obtained from Jackson Animal Laboratories (California). 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 was based on mean body weight and was given in a volume of 200 μl. Peritoneal cavities were lavaged with 5 ml indicator-free and Ca^{2+}-, Mg^{2+}-free Hanks' buffered saline solution (HBSS) that was injected i.p. The abdomen was massaged gently and the peritoneal fluid was removed with a syringe equipped with a 22-gauge needle. Peritoneal exudate cells were pelleted at 200 *g* and washed three times with cold HBSS. The initial supernatant was retained for assay of non-cell-associated liposomal lipid and drug. Peritoneal cells were counted using standard haemacytometer techniques and cell-associated lipid and drug were assayed as indicated below. Where indicated, animals were given (i.p.) L1210 tumor cells, a murine leukemia cell line obtained from the NCI Tumor Repository (Frederick, Md.). Animals were inoculated with 1×10^5 L1210 cells and were injected i.v. with the specified samples 4 days after tumor inoculation. These tumor-bearing animals had no measurable (as judged by weight gain or increase in peritoneal fluid) ascites fluid before day 7.

Liposome preparation. Lipid mixtures were prepared in 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 (100 mg/ml) were formed by hydrating the dried lipid with 300 m*M* citric acid (pH 4.0). The resulting preparation was frozen and thawed five times prior to extrusion ten times through two stacked polycarbonate filters of the indicated pore size [20, 26]. When DSPC or DPPC were employed, the sample and extrusion apparatus (Lipex Biomembranes Inc., Vancouver, B.C., Canada) were heated to 5° C above the thermotropic phase-transition temperature of the phospholipid prior to extrusion [34]. Liposome particle size was determined by quasielastic light-scattering (QELS) measurements (employing a Nicomp 370 particle sizer operating at a wavelength of 632.8 nm).

Doxorubicin encapsulation. Doxorubicin was encapsulated in liposomes in response to transmembrane pH gradients as described previously [27, 31]. 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 and then transferred to a preheated (60° C) vial of doxorubicin, with enough lipid typically being added to achieve a final drug-to-lipid ratio of 0.2 (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 described previously [31]. This procedure resulted in entrapment efficiencies in excess of 98%. 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. Previous studies have demonstrated that this lipid label is non-exchangeable and non-metabolizable [43]. For scintillation counting, samples in HBSS (up to 450 μl) were mixed with 5 ml Pico-Fluor 40 scintillation cocktail and counted employing a Beckman LS3801 scintillation counter.

Doxorubicin levels were determined employing a fluorescent assay procedure. Samples (up to 800 μl) were mixed with 0.1 ml 10% sodium dodecyl sulphate (SDS) and 0.1 ml 10 m*N*H_2SO_4 and, when necessary, were diluted to a final volume of 1 ml with distilled water. Subsequently, 2 ml isopropanol/chloroform (1:1) was added and the sample was mixed vigorously. The resulting solution was frozen at –70° C, thawed and spun at 500 *g* for 3–5 min. The organic phase was carefully removed and the fluorescence of this phase was determined (excitation wavelength, 500 nm; emission wavelength, 550 nm) employing a Shimadzu RF-540 spectrofluorometer. If required, sample volumes were increased or decreased such that the doxorubicin level within the 1-ml aqueous solution fell within the range of the standard curve. The resulting data were converted to doxorubicin fluorescent equivalents derived from a standard curve prepared from doxorubicin. High-performance liquid chromatographic (HPLC) analysis of selected samples was performed to provide an indication of the amount of fluorescence that was due to non-metabolized doxorubicin.

Flow cytometry. Cells isolated from the peritoneal cavity were washed in ice-cold sterile HBSS and resuspended in 1–2 ml HBSS supplemented with 5% fetal bovine serum. The fluorescence of the cells was analyzed using a FACS400 system (Becton-Dickinson) with the laser operating at 488 nm (0.3 W). Doxorubicin fluorescence was detected using the same band-pass filters employed for fluorescein (530 nm).

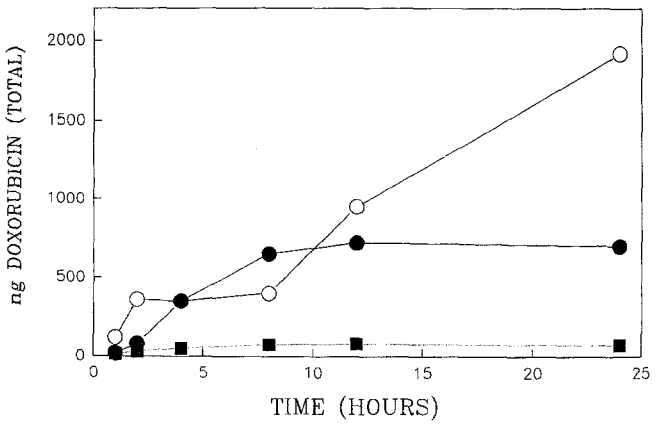

Fig. 1. Quantification of total peritoneal cavity-associated doxorubicin. Following administration of 100-nm DPPC/chol liposomal doxorubicin or free doxorubicin at 20 mg drug/kg the peritoneal cavity was lavaged employing 5 ml HBS. The lavage fluid was collected and cells were pelleted at 200 *g*. The pelleted cells and a portion of the supernatant were assayed for doxorubicin employing standard procedures. The *open circles* represent non-cell-associated doxorubicin from animals receiving liposomal drug, the *filled circles* represent cell-associated doxorubicin from animals given liposomal doxorubicin and the *filled squares* represent cell-associated drug from animals given free drug. There was no detectable level of non-cell-associated drug in animals given free drug. The data represent mean values for drug obtained from at least four mice per time point

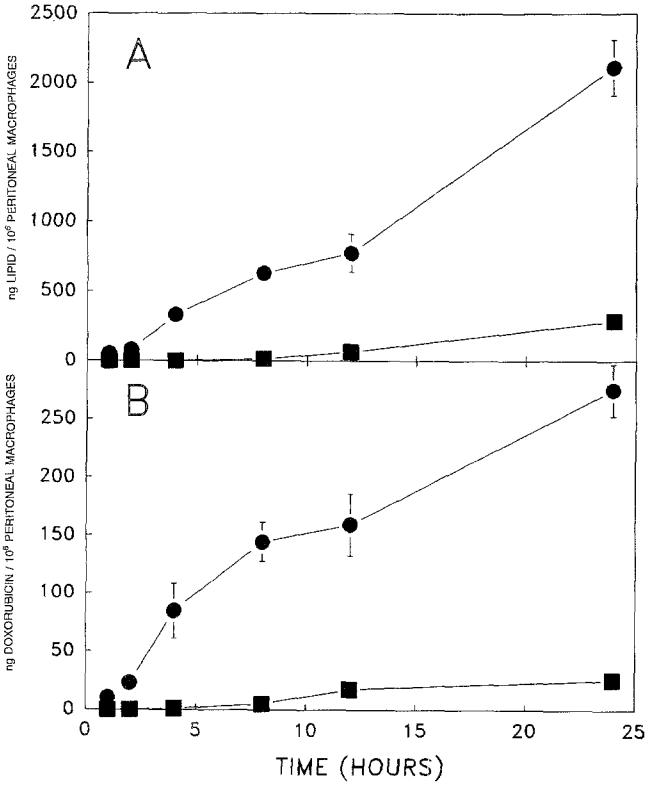

Fig. 2 A, B. The influence of vesicle size on the association of liposomal doxorubicin with peritoneal cells. Animals were given i.v. 100-nm DPPC/chol liposomal doxorubicin (*filled circles*) or 1.0-μm DPPC/chol liposomal doxorubicin (*filled squares*). At the indicated time points, cell-associated lipid (**A**) and doxorubicin (**B**) were determined as described in Materials and methods. The values were derived from at least four mice per time point and represent means ± SEM

Viable cells were separated from non-viable cells on the FACS employing pyridinium iodide, a fluorescent dye exclusion marker.

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

Results

Accumulation of doxorubicin in the peritoneal cavity following i.v. administration of free and liposomal doxorubicin

Many studies have shown that liposomal contents accumulate in sites of tumor growth [11, 14, 15, 37, 39, 41, 45]. The tumor models (L1210 and P388 ascitic tumors) used routinely in our laboratory for evaluation of liposomal anticancer agents are grown within the peritoneal cavity of mice [27, 30]. Furthermore, we have demonstrated using these models that the therapeutic activity of liposomal doxorubicin [27] and liposomal vincristine [30] is very sensitive to the size of the liposomal carrier. Specifically, drug encapsulated in small liposomes, exhibiting mean diameters of approximately 100 nm, is significantly more potent than drug encapsulated in large liposomes (> 200 nm). For these reasons this investigation focused on quantifying the amount of doxorubicin entering the peritoneal cavity following i.v. administration of free and liposomal drug.

The data presented in Fig. 1 clearly demonstrate that significant levels of doxorubicin accumulate in the peritoneal cavity of normal mice following i.v. administration of 100 nm DPPC/chol (55:45) liposomal doxorubicin. Both free and liposomal drug were given at a doxorubicin dose of 20 mg/kg and the liposomes exhibited a drug-to-lipid ratio of 0.2 (wt/wt). The data shown in Fig. 1 represent the total drug level isolated from the peritoneal cavity following a 5-ml lavage with HBSS (see Materials and methods) and illustrate the results obtained for both cell- (filled symbols) and non-cell-associated (open symbols) doxorubicin. Two points are evident from these data. First, the drug levels in the peritoneal cavity following i.v. administration of liposomal drug are as much as 100 times greater than can be achieved with free drug (filled squares). Second, peritoneal cavity-associated doxorubicin in animals treated with free drug is measurable only in pelleted cells. This contrasts with the results obtained following injection of the liposomal drug, whereby significant amounts of non-cell-associated drug are observed.

Peritoneal cell binding of liposomes that have moved from the blood to the peritoneal cavity

Given that free drug is found associated only with resident peritoneal cells, the studies presented herein focus primarily on the association of drug and/or liposomes with these cells. These studies used liposomes labeled with a non-exchangeable, non-metabolizable radiolabeled lipid marker, [^{3}H]-cholesterol hexadecyl ether, to quantitate cell-

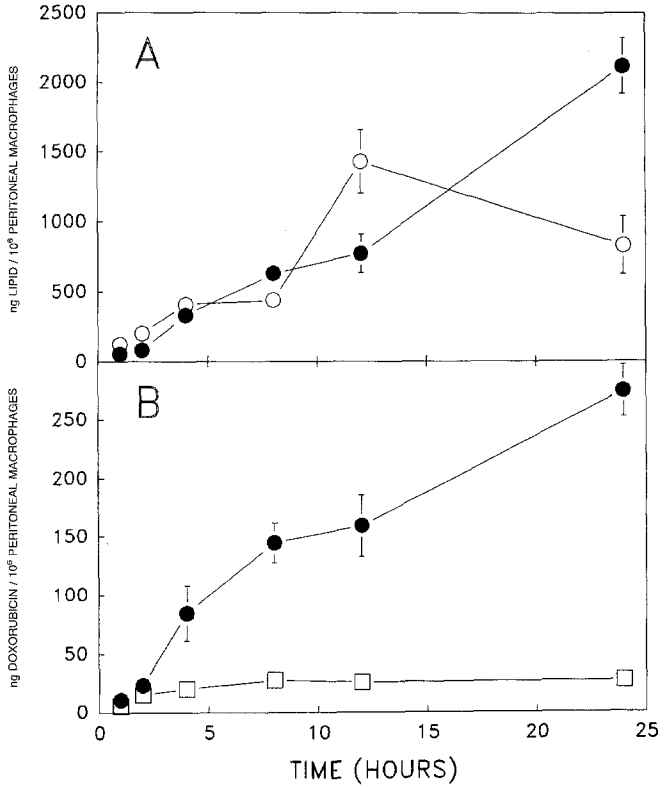

Fig. 3 A, B. Quantification of liposomal lipid (**A**) and doxorubicin (**B**) associated with peritoneal macrophages. Animals were treated with free drug (*open squares*) at a dose of 20 mg/kg, with empty liposomes (*open circles*) at a dose of 100 mg/kg or with 100 nm DPPC/chol liposomal doxorubicin (*filled circles*) at a dose of 20 mg/kg doxorubicin with a drug-to-lipid ratio of 0.2:1 (wt/wt). Values (mean ± SEM) were derived from at least four mice per time point

associated liposome levels. The results (Fig. 2A) demonstrate that liposomal lipid becomes associated with peritoneal cells in a manner that is dependent on vesicle size. Animals were treated i.v. with doxorubicin encapsulated in 0.1-μm (filled circles) or 1.0-μm (filled squares) DPPC/chol (55:45) liposomes that exhibited identical drug-to-lipid ratios. Significantly ($P < 0.001$ at all time points other than 30 min) greater levels of drug (Fig. 2B) and liposomal lipid (Fig. 2A) became cell-associated in animals treated with 0.1-μm liposomal doxorubicin. At the 24-h time point, approximately 10 times more doxorubicin and liposomal lipid was found associated with peritoneal cells from mice injected i.v. with the 0.1-μm liposomes. It should be noted that plasma clearance studies of large (1.0-μm) and small (0.1-μm) DPPC/chol liposomes have been presented in a previous manuscript [3].

Previous results from this laboratory have demonstrated that the circulation lifetime of empty liposomes is significantly shorter than that of identical liposomes containing doxorubicin [3]. It was necessary, therefore, to determine whether liposomal lipid delivery to peritoneal cells occurred only with drug-loaded liposomes. The results shown in Fig. 3A demonstrate that empty liposomes (injected i.v. at a lipid dose of 100 mg/kg) are just as likely to become associated with peritoneal cells as drug-loaded liposomes. The time course for accumulation is identical for the first 8 h. At 24 h, however, a 2-fold greater level of cell-associated lipid is observed for those animals given the drug-loaded carrier. This difference is comparable with the 2- to 3-fold increase in circulating levels of liposomal lipid observed 24 h following i.v. administration of doxorubicin-loaded 0.1-μm DPPC/chol liposomes as compared with empty liposomes [3].

Distribution of doxorubicin in isolated peritoneal cells

Since doxorubicin is a fluorescent molecule, the distribution of drug associated with isolated peritoneal cells can readily be determined using fluorescent microscopy and flow cytometry. Figure 4 includes representative photomicrographs of peritoneal cells obtained from mice 1 day after i.v. administration of free doxorubicin (Fig. 4A), 1.0-μm DPPC/chol (55:45) liposomal doxorubicin (Fig. 4B) and 0.1-μm DPPC/chol (55:45) liposomal doxorubicin (Fig. 4C, D). A significant proportion of peritoneal cells isolated from animals given 0.1-μm liposomal doxorubicin were clearly fluorescent.

Flow cytometric analysis of peritoneal cells isolated from animals given (i.v.) 0.1-μm liposomal doxorubicin (Fig. 5C) indicated that fluorescence was primarily associated with a single cell population. As shown in Fig. 5A, flow cytometric analysis of peritoneal cells isolated from control animals revealed two distinct cell populations on the basis of orthogonal light-scatter characteristics. These were identified as macrophages (large granular cells) and lymphocytes (small cells). The proportion of each population (approximately 40% for macrophages and 60% for lymphocytes) was consistent with the results obtained in other laboratories [33]. After compensation for control cell autofluorescence, the flow cytometry data indicated that only the large cell population (macrophages) accumulated doxorubicin.

Influence of lipid composition on doxorubicin accumulation in peritoneal cells

The results presented to this point demonstrate that liposomal lipid and doxorubicin accumulate in cells residing in the peritoneal cavity following i.v. administration of 0.1-μm DPPC/chol liposomal doxorubicin. The level of drug achieved in the peritoneal cells is 10 times greater than that achieved with free doxorubicin or doxorubicin encapsulated in large liposomes. It is known that liposomal lipid composition strongly influences doxorubicin retention in vivo [3]. The influence of lipid composition on the level of peritoneal cell-associated doxorubicin achieved following i.v. injection of 0.1-μm (filled bars) and 1.0-μm (slashed bars) liposomal doxorubicin is shown in Fig. 6. These liposomal formulations were prepared employing vesicles composed of the indicated phosphatidycholine (55 mol%) plus cholesterol (45 mol%), and all were given at identical drug (20 mg/kg) and lipid (100 mg/kg) doses.

The data indicate that i.v. administration of liposomal doxorubicin prepared with small (0.1-μm) vesicles composed of saturated phospholipids (DPPC or DSPC) results in greater delivery of drug to peritoneal cells. As compared

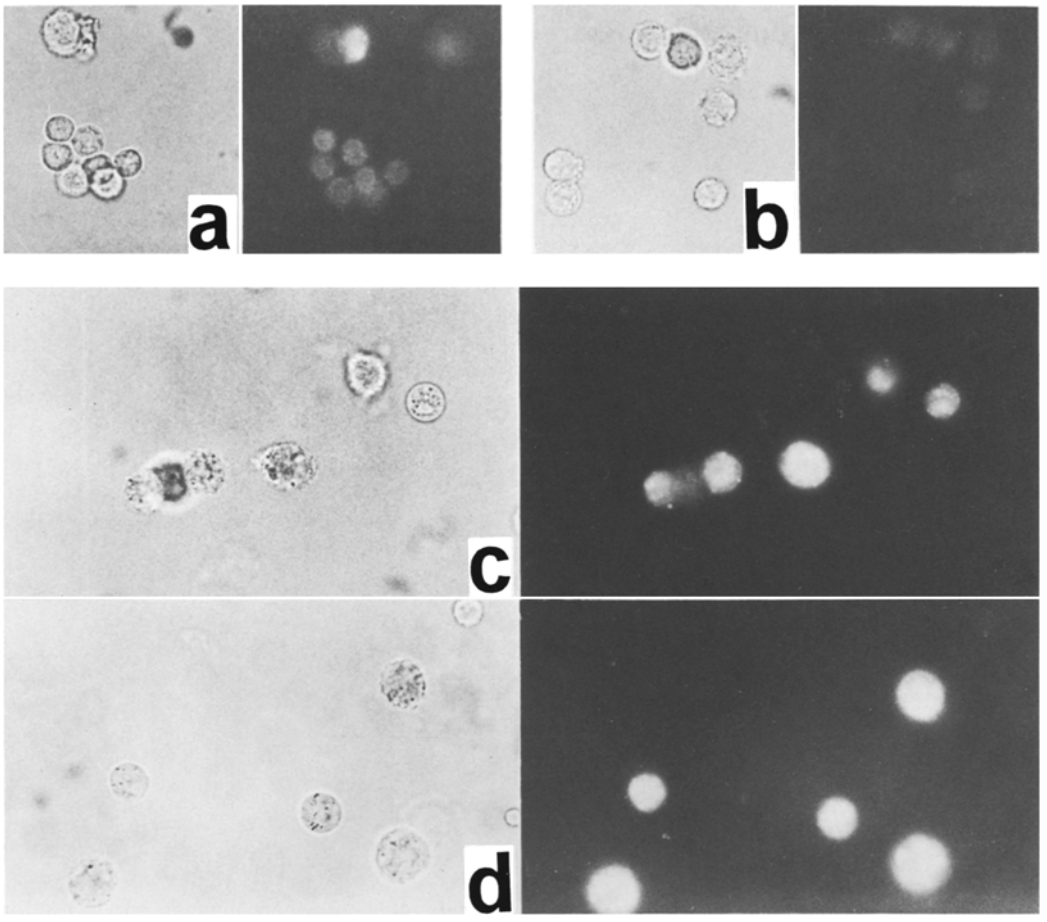

Fig. 4 a–d. Photomicrographs of peritoneal cells obtained from animals treated i.v. (24 h previously) with free doxorubicin (**a**), 1.0-μm DPPC/chol liposomal doxorubicin (**b**) and 0.1-μm DPPC/chol liposomal doxorubicin (**c**, **d**). Corresponding fluorescent micrographs taken employing a Wild Leitz microscope with epifluorescence are presented

with free drug, 5, 10 and 20 times more doxorubicin became associated with peritoneal cells following injection of 0.1-μm liposomes composed of EPC/chol (unsaturated PC), DPPC/chol and DSPC/chol, respectively. These differences in drug levels can be attributed to differences in rates of drug release from liposomes and are evident from estimation of cell-associated drug-to-liposomal lipid ratio (wt/wt). For peritoneal cells isolated from animals injected with EPC/chol, DPPC/chol and DSPC/chol, the drug-to-liposomal lipid ratio obtained was 0.08, 0.12 and 0.2, respectively (data not shown). Doxorubicin could not be detected in peritoneal cells from animals injected with large (1.0-μm) DSPC/chol liposomal doxorubicin.

Suppression of peritoneal macrophages following i.v. administration of free and liposomal doxorubicin

While pursuing the studies summarized above, we noted that 24 h after animals had been given 0.1-μm DSPC/chol liposomal doxorubicin there was an approximately 50% reduction in the peritoneal cell population as compared with animals injected with free drug. This biological effect was consistent with the observation that more doxorubicin, a potent cytotoxic drug, was associated with peritoneal macrophages when given in 0.1-μm liposomes. The extent of peritoneal macrophage suppression obtained following i.v. administration of free or liposomal drug was characterized further and the results are shown in Fig. 7. It was decided on the basis of the data presented in Fig. 6 that studies on liposomal doxorubicin-induced suppression of peritoneal macrophages would be determined for 0.1- and 1.0-μm liposomes composed of EPC/chol (leaky, Fig. 7A) or DSPC/chol (non-leaky, Fig. 7B). Animals were treated with liposomes exhibiting a drug-to-lipid ratio (wt/wt) of 0.2 and at a drug dose of 20 mg/kg. This drug dose is the maximum tolerated dose (MTD) for free drug in DBA mice. The MTD in DBA mice for doxorubicin entrapped in 0.1-μm liposomes composed of EPC/chol and DSPC/chol is 30 and >60 mg/kg, respectively (unpublished observations).

The time course chosen for the studies illustrated in Fig. 7 was based on other studies indicating that the turnover rate of murine peritoneal macrophages is approximately 15 days [13]. Animals injected with free drug (open circles, Fig. 7A, B) showed a gradual decline in the number of peritoneal macrophages. A nadir was reached on day 3, when a 65% reduction in the number of peritoneal macrophages was observed. Recovery to control values was first achieved 14 days after drug administration. In comparison with free drug, doxorubicin entrapped in small (0.1-μm) liposomes composed of EPC/chol or DSPC/chol was significantly more effective in suppressing peritoneal macrophages. As was the case for free drug, a nadir was obtained on day 3; however, the extent of suppression was greater (80%–90% as compared with untreated controls) and the level of suppression was maintained for a longer

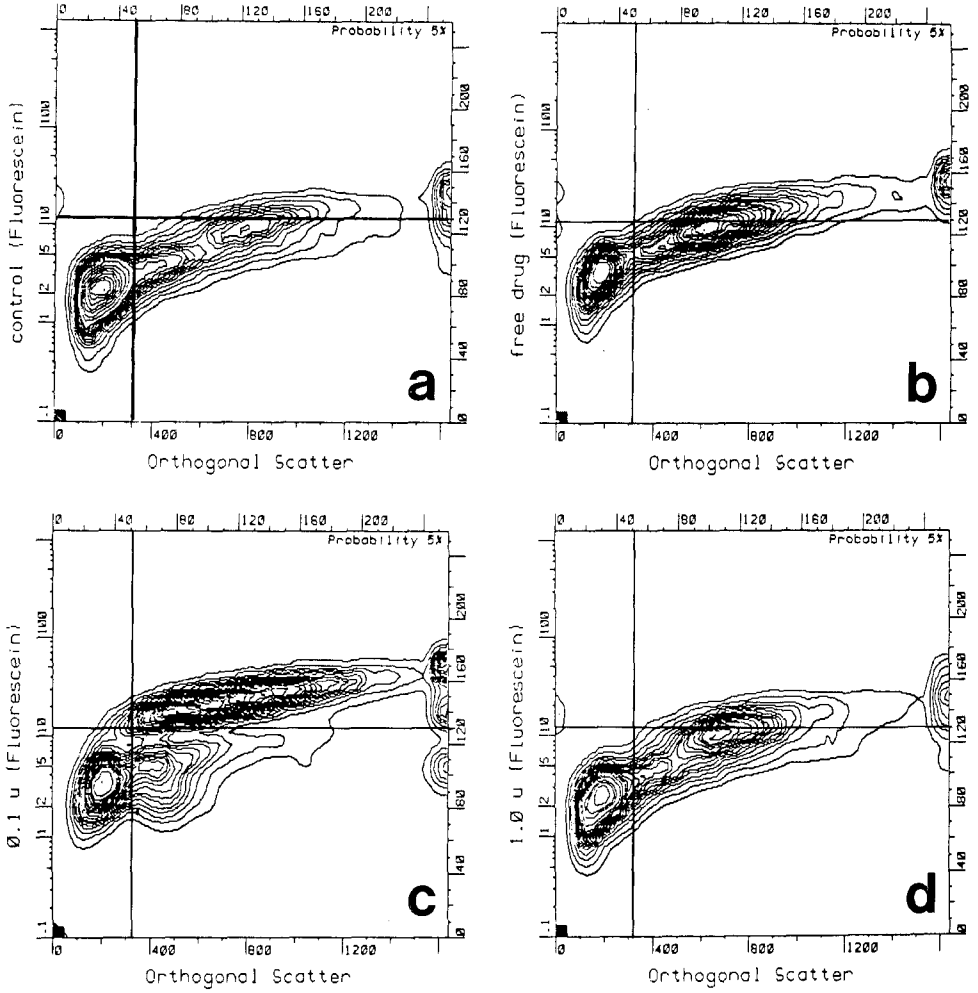

Fig. 5 a–d. Bivariant flow cytometric analysis of peritoneal cells comparing fluorescent intensity (*ordinate*) against orthogonal scatter (*abscissa*). The upper left hand figure (**a**) was obtained from cells isolated from untreated animals. The *vertical line* was used to indicate separation between small cells (on the *left*) and large cells (on the *right*), which were tentatively identified as lymphocytes and macrophages, respectively. The *horizontal line* was used to separate autofluorescences (*below the line*) and doxorubicin-associated fluorescence (*above the line*). The remaining figures were obtained for cells from animals given (i.v., 20 mg/kg) free doxorubicin (**b**), 0.1-μm DPPC/chol liposomal doxorubicin (**c**) and 1.0-μm DPPC/Chol liposomal doxorubicin (**d**)

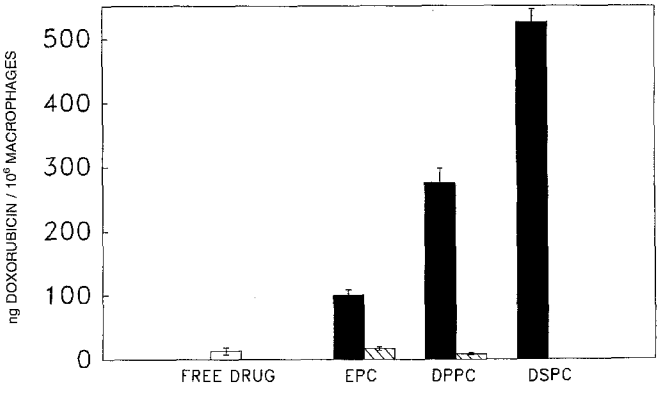

Fig. 6. Influence of lipid composition on the level of peritoneal macrophage-associated doxorubicin measured 24 h after administration of 100-nm liposomal doxorubicin (*filled bars*) and 1.0-μm liposomal doxorubicin (*slashed bars*). Liposomal doxorubicin was prepared employing vesicles composed of the indicated phosphatidylcholine (55 mol%) and cholesterol (45 mol%). Peritoneal cells were isolated by lavage as described in Materials and methods and values (mean ± SEM) were obtained from at least four mice per group

time (beyond day 7). By day 14, animals injected with 0.1-μm EPC/chol liposomal doxorubicin had populations of peritoneal macrophages comparable with those given free drug. In contrast, doxorubicin entrapped in 0.1-μm DSPC/chol had a prolonged effect, with cell numbers on day 14 being 60% lower than those in untreated animals. This degree of peritoneal macrophage suppression was significantly greater ($P < 0.01$) than that observed in animals given free drug. The data shown in Fig. 7 indicate that doxorubicin entrapped in 1.0-μm liposomes was much less effective at inducing peritoneal cell suppression than was the free drug (filled squares).

Accumulation of liposomal doxorubicin in the peritoneal cavity of mice with ascitic L1210 tumors

It has been demonstrated elsewhere that blood vessels lining the peritoneum become hyperpermeable to macromolecules in mice bearing ascitic tumors [32]. For this reason, movement of free and liposomal doxorubicin from

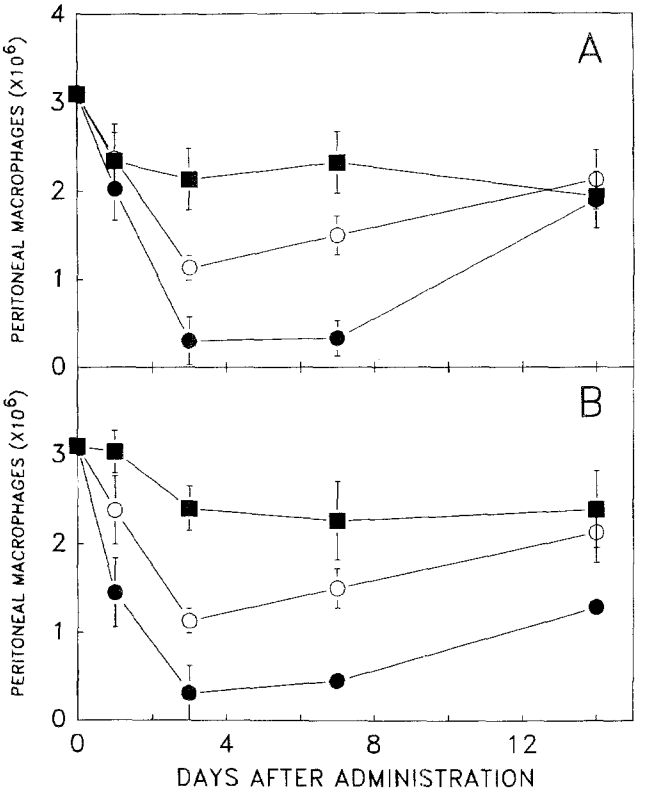

Fig. 7 A, B. The influence of free doxorubicin (*open circles*), EPC/chol liposomal doxorubicin (**A**) and DSPC/chol liposomal doxorubicin (**B**) on the number of peritoneal macrophages isolated after a single peritoneal lavage with HBS (5 ml/cavity) on the specified day following i.v. administration of 100-nm (*solid circles*) or 1000-nm liposomal doxorubicin (*solid squares*). All animals were given a doxorubicin dose of 20 mg/kg. Values (mean ±SD) were obtained from four mice per time point

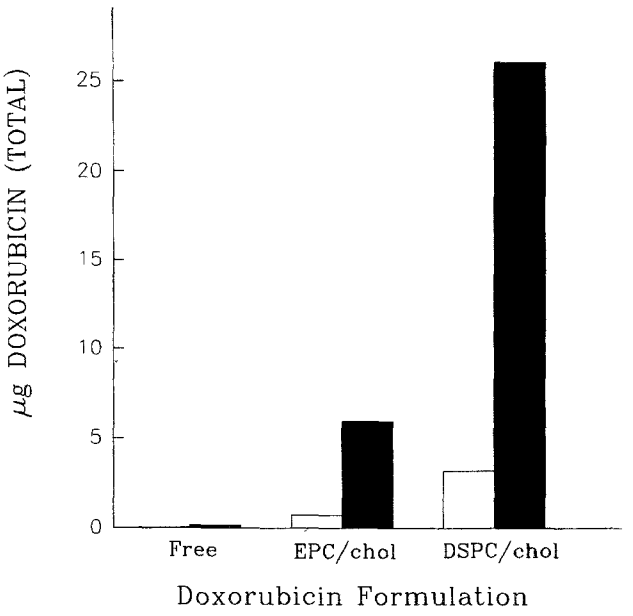

Fig. 8. Amount of doxorubicin recovered from the peritoneal cavity of mice with (*filled bars*) and without (*open bars*) an established L1210 ascitic tumor (see Materials and methods). Mice were given the specified formulation of doxorubicin by i.v. administration and the level of drug in the peritoneal cavity was measured 24 h after drug administration. The data represent the mean values for cell-associated plus non-cell-associated drug obtained from at least four mice per group

the blood compartment to the peritoneal cavity was determined in mice with established L1210 ascitic tumors (Fig. 8). Tumors were initiated by i.p. injection of 10^5 L1210 cells. Free and liposomal drug were given on day 4 and the level of drug within the peritoneal cavity was assayed 24 h after drug administration. This time course was selected for the following reasons: (1) the number of cells in the peritoneal cavity on day 5 ($80–120 \times 10^6$ cells) was significantly greater than that in animals without tumors ($3–5 \times 10^6$ cells), indicative of tumor progression; (2) there was no measurable volume of ascitic fluid in the peritoneal cavity 5 days after tumor inoculation as judged by weight increase or changes in the volume of fluid recovered after a 5-ml lavage (see Materials and methods); and (3) if the tumor progressed beyond 7 days, significant quantities of ascitic fluid developed and this fluid was contaminated with red cells.

The amount of doxorubicin recovered after peritoneal lavage of animals given (i.v.) free or liposomal doxorubicin increased substantially when the animals had established L1210 tumors (Fig. 8). At 1 day after i.v. injection of doxorubicin entrapped in DSPC/chol (100-nm) liposomes, more than 5% of the injected drug dose was recovered in the peritoneal cavity of the tumor-bearing animals. This level of drug was almost 100 times that achieved following injection of free doxorubicin. It is noteworthy that the increase in peritoneal cavity-associated drug levels in tumor-bearing animals that received liposomal doxorubicin is primarily a consequence of increases in non-cell-associated drug. For mice given doxorubicin encapsulated in 100-nm DSPC/chol liposomal doxorubicin, more than 95% of the total drug levels obtained were due to non-cell-associated drug. As shown in Fig. 9, there was a significant decrease in the amount of cell-associated drug as calculated on the basis of nanograms of drug per 10^6 peritoneal cells when those cells were derived from tumor-bearing animals. The total number of cells isolated from animals with tumors, however, was greater than those obtained from tumor-free control animals (Fig. 10). The cell number obtained following lavage of tumor-bearing animals depended on drug treatment. A 50% reduction in peritoneal cells was observed in animals given free doxorubicin or doxorubicin encapsulated in EPC/chol liposomes. In contrast, there was no change in the cell number obtained following lavage of animals given DSPC/chol liposomal doxorubicin.

Discussion

The results presented herein demonstrate that following i.v. administration, liposomal doxorubicin can accumulate within the peritoneal cavity of mice. This accumulation process is shown to be dependent on the characteristics of the liposomes employed. The results indicate that optimal delivery occurs via the use of small liposomes (exhibiting mean diameters of approximately 0.1-μm), which retain encapsulated contents and exhibit extended circulation

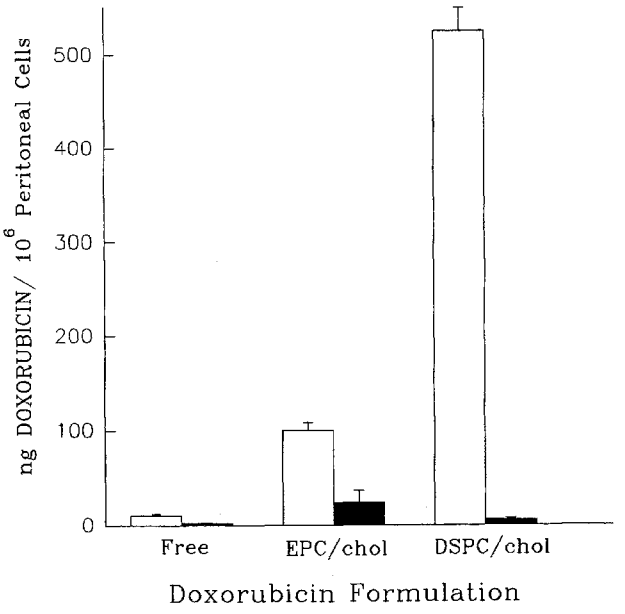

Fig. 9. Levels of peritoneal cell-associated doxorubicin measured 24 h after i.v. administration of the specified drug formulation to animals with (*filled bars*) and without (*open bars*) an established L1210 tumor (see Materials and methods). Peritoneal cells were isolated by lavage and doxorubicin levels (±SEM) were obtained from at least four mice per group

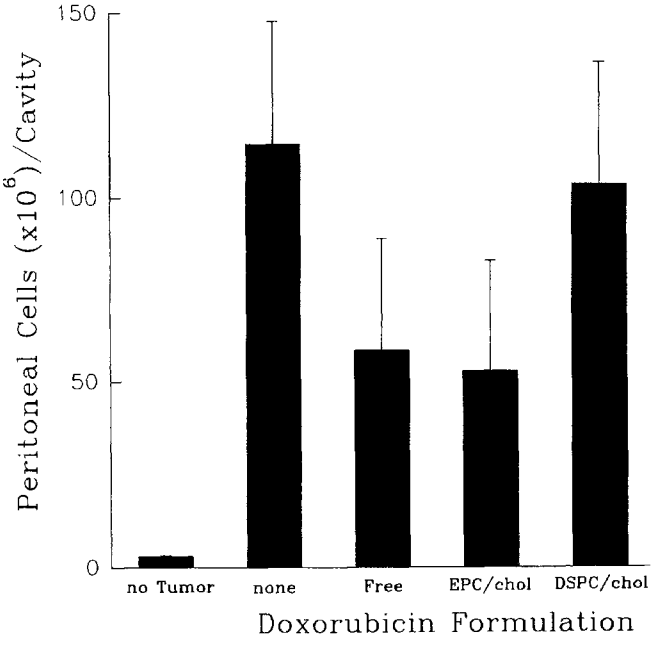

Fig. 10. The number of peritoneal cells isolated from mice with established L1210 tumors (see Materials and methods) was determined after lavage of animals given the specified doxorubicin formulation 1 day prior to the isolation of peritoneal cells. The data were obtained from 4 mice per group (those obtained in the absence of tumor were based on a sampling of 16 mice) and values shown are means ±SD

lifetimes. The level of uptake is further enhanced in animals that have an established tumor localized in the peritoneal cavity. It is suggested that the peritoneal cavity can serve as an appropriate extravascular site for characterizing and optimizing the passive targeting properties of liposomes. The discussion will focus on how this extravasation model may be of use in developing a better understanding of the biological factors that promote transfer of liposomes from the blood compartment to an extravascular site.

A central question regarding the delivery of liposomal anticancer agents to tumors concerns what role the tumor vascular structure plays in promoting leakage of liposomes from the blood compartment to an extravascular site in the tumor and whether one can optimize liposomal delivery systems such that delivery to an extravascular site is maximized. One approach to this question is to study extravasation in the absence of disease. Other investigators have characterized influx of vascular components (serum proteins and other macromolecules) into the peritoneal cavity to model extravasation kinetics as a function of the components' molecular size and physical and chemical characteristics (e.g. charge, shape, presence or absence of carbohydrate moieties). The blood vessels lining the peritoneum are relatively intact in disease-free animals, and influx into the peritoneal cavity requires that barriers such as the vascular endothelium, with intact basement membranes, be traversed. Transport of macromolecules ranging in size from 5 to >700 kDa into the peritoneal cavity of normal mice has been observed [28, 29]. The results reported herein suggest that liposomes exhibiting mean diameters of approximately 100 nm can also gain access to the peritoneal cavity following i.v. administration. In fact, the present data (Figs. 2, 3) indicate that drug accumulation is due primarily to drug-loaded liposomes that have left the circulation.

Several possible mechanisms can be postulated to explain how liposomes leave the circulation to access an extravascular site in disease-free control animals. Macromolecule and liposome movement through pores or fenestration present in certain microvascular systems will be a contributing factor. Assuming that the blood vessels lining the peritoneum are intact and do not exhibit a leaky endothelium [32], one must consider mechanisms of extravasation that involve the endothelial cells or cells that are capable of traversing the endothelial barrier. Endothelial cells may play a direct role in the movement of liposomal carriers via transcytosis. Alternatively, liposome extravasation may involve movement of monocytes from the circulation to the peritoneal cavity. It has been demonstrated, for example, that monocytes can phagocytose particulates in the circulation [9, 12, 25]. Subsequently these cells may migrate to extravascular sites such as the peritoneal cavity. The data presented in this report would suggest that this mechanism is unlikely to be important. In particular, large liposomes that are more readily accumulated in phagocytic cells do not accumulate in the peritoneal cavity.

Modes of extravasation that involve endothelial cell transcytosis and/or regions of vascular hyperpermeability would be facilitated in regions of disease progression, such as a tumor. Tumor cells, for example, release factors that induce pore formation between and promote transcytosis through endothelial cells [5, 6, 22]. It is demonstrated in this report that liposome extravasation into the peritoneal

cavity is enhanced by a factor of at least 10 when the animal has an established peritoneal tumor.

An important advantage of using the peritoneal cavity as a model for studying extravasation concerns the ability to assess the distribution of drug and liposomes within specific cell populations. The results presented herein demonstrate that liposomes and associated drug that enter the peritoneal cavity from the circulation interact with cells in the peritoneal cavity (Figs. 2, 3). Flow cytometric analysis of these cells (Fig. 5) indicates, however, that liposomal drug associates primarily with only one cell population, the peritoneal macrophages. Lymphocytes residing in the peritoneal cavity do not appear to accumulate liposomal drug. A similar analysis of cells isolated from animals bearing L1210 leukemia was more ambiguous, since light-scatter data did not resolve distinct cell populations (data not shown). The present data, however, show that most of the liposomal doxorubicin within the ascitic tumor is not associated with the L1210 tumor cells.

In conclusion, it is believed that research focused on optimization of liposomal anticancer drugs and, in particular, development of ligand-directed targeted liposomal carriers must take into consideration the mechanisms responsible for movement of liposomes from the blood (or plasma) compartment to an extravascular site. These mechanisms are poorly understood at present. The approach described herein provides a convenient, easily assessable model for studying liposome extravasation. Transport from the blood into the peritoneum can be studied in disease-free animals and characterization of the liposomes that have entered this site is relatively simple. The results presented herein illustrate the importance of liposome variables such as vesicle size, lipid composition and lipid dose with respect to maximizing delivery to a site outside the blood compartment. Furthermore, the peritoneal cavity model used in the present study to assess extravasation of circulating liposomes should be ideal for identifying and characterizing biological factors that may promote liposome accumulation within a target extravascular site, such as a solid tumor.

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