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ORIGINAL ARTICLE

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Intratumor distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes

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Abstract *Purpose*: A pharmacological evaluation of an egg phosphatidylcholine/cholesterol (55:45 mole ratio, EPC/Chol) liposome doxorubicin formulation was carried out. The objective was to define liposomal lipid and drug distribution within sites of tumor growth following intravenous (i.v.) administration to female BDF1 mice bearing either Lewis lung carcinoma, B16/BL6 melanoma, or L1210 ascitic tumors. *Methods*: Mice were injected i.v. with EPC/Chol liposomal doxorubicin, and plasma and tumor levels of lipid and drug were determined 1, 4 and 24 h later with radiolabeled lipid and fluorimetry or fluorescence microscopy, respectively. In addition, single-cell suspensions of the Lewis lung and B16/BL6 tumors were prepared and the presence of macrophages was determined with an FITC-labeled rat antimouse CD11b (MAC-1) antibody. *Results*: For mice bearing the Lewis lung solid tumors, there was a time-dependent accumulation of liposomal lipid, with a plateau of approximately 500 μg lipid/g tumor at 48 h. In contrast, the apparent plateau (μg doxorubicin/g tumor) for doxorubicin was achieved at 1 h and remained constant over a 72-h time course. In comparison with free drug administered at the maximum tolerated dose (MTD, 20 mg/kg) doxorubicin levels in tumors were two- to threefold greater when the drug was administered in liposomal form. The increase in drug delivery was comparable for both solid tumors. With animals bearing the L1210 ascitic tumor, drug exposure was as much as ten times greater (in comparison with free drug) when doxorubicin was administered in liposomes. An evaluation of single-cell suspensions prepared from the two solid tumors suggested that more than 98% of the tumor-associated drug and liposomal lipid was not tumor cell-associated. Histological studies with the Lewis lung carcinoma, however, revealed that a proportion of the drug did colocalize with tumor-associated macrophages. Analysis of cells obtained from mice bearing ascitic tumors showed that more than 80% of the cell-associated drug could be removed by procedures designed to remove adherent cells. *Conclusion*: The results summarized here suggest drug concentrations within a solid tumor, such as the Lewis lung carcinoma, are constant over time when the drug is given in a "leaky" EPC/Chol formulation. The results also suggest that liposomal lipid within sites of tumor growth is primarily localized within the interstitial spaces or tumor-associated macrophages.

Key words Liposomes · Doxorubicin · Extravasation

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Introduction

The principal benefit achieved following administration of anticancer drugs encapsulated in liposomes is carrier-mediated delivery of drug to the site of disease [1, 7, 8, 22]. Considering the important role of carrier-mediated drug delivery in defining therapeutic activity [20], it can be reasoned that high drug-to-lipid ratios and enhanced drug retention characteristics are required to ensure that the greatest quantity of drug is delivered via liposomes to extravascular sites. The problem with this rationale is that drug must be released from the liposomes in order to exert its effect [20]. In this regard we believe that liposomes can function as delivery systems, where cell-specific transfer is the goal, and good drug retention characteristics are required. Alternatively, liposomes can act as sustained release systems, where optimal drug release characteristics govern therapeutic activity. For nontargeted liposomal carriers it is anticipated that the latter is the most important function, and it is important to establish a balance between drug

release from liposomes that have extravasated and retention of drug during the time period required for extravasation to occur.

The primary objective of the studies summarized here was to define the intratumor distribution of drug following intravenous (i.v.) administration of an egg phosphatidylcholine/cholesterol (EPC/Chol) liposomal doxorubicin formulation. The parameters assessed included measurements of drug and liposomal lipid in the plasma and at sites of tumor growth. EPC/Chol was selected because it has been established that drug release from these liposomes is significantly faster than from liposomes prepared with saturated phosphatidylcholine species such as DSPC and DPPC [4, 15]. Further, previous studies have shown that the therapeutic activity of these latter formulations, when administered at equivalent doses to the EPC/Chol formulation, is significantly less [18]. It is anticipated that enhanced activity is a consequence of enhanced drug release from the EPC/Chol formulation and we suggest this should be reflected in improved drug bioavailability and increases in drug binding to tumor-associated cells.

The second objective of these studies was to define the intratumor distribution of liposomal lipid following i.v. administration of an EPC/Chol liposomal doxorubicin formulation. It can be speculated that along with carrier-mediated increases in regional drug localization, further optimization in therapeutic activity would be achieved using carriers that specifically bind to tumor cells (targeting). Here, the long-term aim is to define formulation characteristics that facilitate direct interaction of liposomal carriers with cells within the tumor. We believe, however, that if targeting is to be of potential therapeutic value it is essential to develop a carrier with targeting features that do not interfere with its tendency to move from the blood compartment to an extravascular site [5, 28]. Within this context it is just as important to determine the availability of extravasated liposomes for targeting, since nonspecific association of liposomes with nontumor cells such as tumor-associated macrophages or endothelial cells of the tumor vasculature will interfere with the potential for liposomes to efficiently bind the specific tumor cell population.

Materials and methods

Drugs and chemicals

Liposomal doxorubicin was provided as a kit by The Liposome Company, (Princeton, N.J.). It included EPC/Chol liposomes (100 mg/ml, 120 nm mean diameter as determined by quasielastic light scattering, QELS), 0.1 *M* sodium carbonate, and doxorubicin (adriamycin, 10 mg vial with 60 mg lactose and 1 mg methylparaben). EPC was purchased from Avanti Polar Lipids (Birmingham, Ala.). Cholesterol and deoxyribonuclease I (DNase I) were obtained from Sigma (St. Louis, Mo.). Radiolabeled [^{3}H]-cholesteryl hexadecyl ether (^{3}H-CHE) was purchased from Amersham (Oakville, Ont.) and doxorubicin from Adria Laboratories (Mississauga, Ont.). FITC-labeled rat antimouse CD11b (MAC-1) antibody was purchased from PharMingen (San Diego, Calif.) and Tissue-Tek OCT compound (10% polyvinyl alcohol, 4% polyethylene glycol, and 86% nonreactive ingredients) from Miles (Elkhart, Ind.). RPMI-1640 and Hank's balanced salt solution (HBSS) were purchased from StemCell Technologies (Vancouver, B.C.). Fetal bovine serum (FBS) and trypsin were from ICN Biomedicals (Mississauga, Ont.). Pico-Fluor 40 was from Canberra Packard (Meriden, Ct.) and microtainer EDTA-coated tubes from Becton Dickinson (Mississauga, Ont.).

Preparation of liposomal doxorubicin

EPC/Chol liposomal doxorubicin was prepared as outlined in directions included as part of a kit that consisted of EPC/Chol liposomes (100 mg/ml solution in 300 m*M* citrate buffer, pH 4.0), sodium carbonate (53 mg/ml), adriamycin (10 mg doxorubicin), and 0.9% sodium chloride. The reconstitution procedure consisted of three steps. First, 1.9 ml liposome solution was mixed with 1.2 ml sodium carbonate. Second, 4.4 ml 0.9% sodium chloride was added to the 10 mg vial of doxorubicin. Finally, 0.6 ml liposomes (EPC/Chol/sodium carbonate) was injected into the vial of doxorubicin. To encapsulate doxorubicin the mixture was immediately heated in a water bath at 60 °C for 10 min with occasional mixing. When radiolabeled liposomes were required for pharmacokinetic studies, 200 µl of the radiolabeled liposomes (see below) was added to the 2.1-ml vial of EPC/Chol liposomes prior to initiation of the reconstitution procedure. The final lipid concentration for this vial was less than 0.5% greater than the original liposome supply.

Production of tracer liposomes

To follow liposome distribution following i.v. administration, labeled (^{3}H-CHE) EPC/Chol liposomes (100 nm) were used. These liposomes were prepared in a manner identical to that used for the preparation of the kit. The methods involve extrusion of multilamellar vesicles through polycarbonate filters exhibiting 100-nm pores as described by Hope et al. [11]. Lipid mixtures consisting of EPC and cholesterol (55:45 mole%) were dissolved in chloroform with the addition of 100 µCi ^{3}H-CHE/10 mg total lipid and concentrated to a homogeneous lipid film under a stream of nitrogen gas. The lipid film was placed under high vacuum for at least 4 h prior to hydration at room temperature with 50 µl 300 m*M* citrate buffer, pH 4.0. Premade EPC/Chol liposomes (950 µl, obtained from the kit described above) were added to the resulting solution to a final lipid concentration of 105 mg/ml. The sample was frozen and thawed five times [17] before extruding ten times through three stacked 100-nm polycarbonate filters (Poretics, Livermore, Calif.) employing an extrusion device (Lipex Biomembranes, Vancouver, B.C.). The resulting liposomes were sized using a Pacific Scientific Nicomp 270 submicron particle sizer operating at a laser wavelength of 632.8 nm (Santa Barbara, Calif.). The liposomes exhibited a mean size distribution identical to that of the clinical supplies. The resulting radiolabeled liposomes were filter sterilized through a 0.22 µm filter and placed into a sterile vial.

Plasma elimination and tissue distribution studies

Female BDF1 mice (18–22 g) were obtained from Charles River Canada (St. Constant, Que.). Mice were weighed and organized into appropriate groups 1 day prior to initiation of the study. For treatments, mice were given a single bolus lateral tail vein injection of the indicated doxorubicin formulations. At 1, 4 and 24 h after administration, mice from each group were sacrificed by CO_2 asphyxiation. Blood was immediately removed by cardiac puncture and collected into an EDTA-coated microtainer tube. The samples were centrifuged at 500 *g* in a clinical bench-top centrifuge for 10 min. Plasma was removed and placed into an Eppendorf tube prior to analysis of lipid and/or doxorubicin (see below). Where indicated, tissue samples were collected in preweighed glass tubes. The weight of tissue was then determined prior to freezing at −70 °C. Frozen tissue samples were homogenized in distilled water with a Kinematica Polytron tissue homogenizer (Switzerland) to a 10% homogenate (w/v). If required, tumors were also prepared for fluorescent microscopy (see below).

Quantitation of liposomal lipid and doxorubicin

Liposomal lipid was quantified by employing the nonexchangeable and nonmetabolizable lipid marker, ^{3}H-CHE [4]. Tracer liposomes were mixed with EPC/Chol liposomes to a known specific activity prior to injection. Upon animal sacrifice, at the indicated time-points, selected tissues were isolated and prepared as 10% homogenates, and 200 µl aliquots of homogenate were assayed as previously outlined [4, 18]. For plasma samples, 100–200 µl was added directly to Pico-Fluor 40 scintillant. Samples were subsequently assayed for radioactivity using a Canberra Packard 1900TR Liquid Scintillation Counter (Meriden, Ct.). Background radioactivity was determined using samples derived from control tissues and plasma obtained from untreated mice.

Doxorubicin was quantified using a modified extraction assay as previously outlined [4, 18]. A standard doxorubicin curve was prepared in control tissue homogenates employing an identical extraction procedure to that described above. Drug levels were estimated on the basis of doxorubicin fluorescent equivalents, but analysis of selected plasma samples by HPLC indicated that >90% of the fluorescence was derived from doxorubicin. The extraction efficiency for each sample was dependent on the tissue type, being typically more than 85%. Distinguishing encapsulated doxorubicin from nonencapsulated doxorubicin was not undertaken as the total levels of nonencapsulated doxorubicin with EPC/Chol liposomes are small [25].

Tumors

Lewis lung carcinoma and B16/BL6 melanoma were from the NCI Tumor Repository (Fredrick, Md.). The L1210 lymphocytic leukemia was from the ATCC (Rockville, Md.). Subcutaneous injection of Lewis lung cells typically required 10 days growth and B16/BL6 cells required 2 weeks growth before an optimal tumor size of 1–2 mm^3 was reached. The ascitic L1210 cells, injected intraperitoneally (i.p.), required 1 week of growth before experiments were initiated.

Tumor fixation and staining

Isolated solid tumors were collected in phosphate-buffered saline (PBS) at 4 °C. Tumors were subsequently fixed with a 3% paraformaldehyde solution in PBS at 4 °C for 30 min. Tumors were washed with PBS and immersed in increasing concentrations of sucrose for 20 min: 10% sucrose PBS, 15% sucrose PBS, and 15% sucrose PBS containing OCT compound (1/1 v/v). The processed tissue was embedded in OCT compound and frozen in liquid nitrogen. Sections (5 µm) were prepared on a Leica Frigocut 2800 E microtome (Germany), placed on a slide, and used for antibody staining. The sections were washed three times in PBS and nonspecific antigens were blocked with 0.02% bovine serum albumin (BSA) for 30 min. FITC-labeled MAC-1 (macrophage-specific antibody) was added at a 1:100 dilution and incubated for 30 min at room temperature in a humid chamber. The sections were washed with distilled water to remove salt and mounted on a microscope slide. Phase contrast and fluorescent microscopy were performed with a Leitz Dialux microscope and Orthomat microscope camera (Germany).

Peritoneal lavage and removal of adherent cells

Mice bearing ascitic tumors were asphyxiated with CO_2 and the peritoneum was carefully exposed (without rupturing the membrane) to reveal the peritoneal cavity. HBSS (5 ml) was subsequently injected along the midline into the peritoneal cavity with a 28 G needle and the peritoneum was gently massaged with a pair of curved forceps. Peritoneal fluid was removed with a 20 G needle and transferred to a 15-ml tube (polypropylene) and centrifuged at 500 *g* for 10 min to pellet the cells. The supernatant was discarded and the peritoneal cells were resuspended in indicator-free HBSS supplemented with 10% FBS. A portion of the isolated cells was analyzed by flow cytometry and a portion was assayed for liposomal lipid and doxorubicin as indicated previously. The remaining sample was transferred to culture flasks and incubated for 2 h at 37 °C in a CO_2 incubator. Nonadherent cells were collected, counted, and washed extensively with medium and analyzed by flow cytometry. The levels of doxorubicin and liposomal lipid associated with these nonadherent cells were determined as outlined previously.

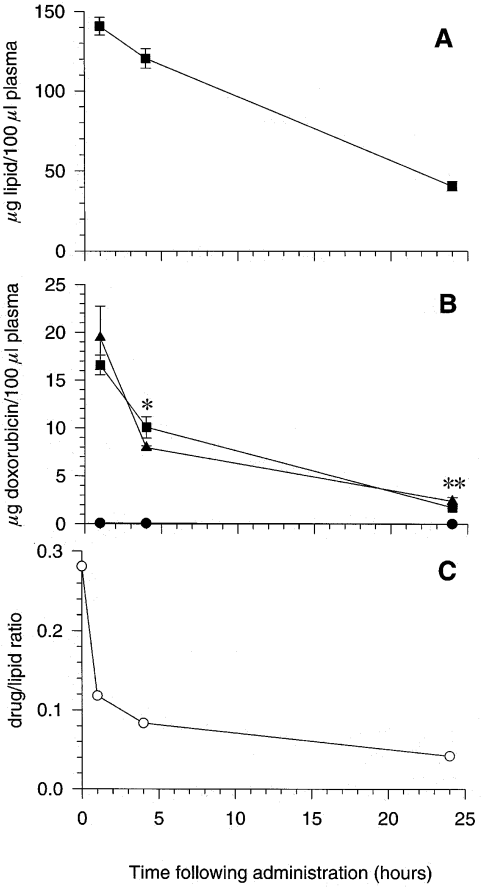

Fig. 1A–C Plasma clearance of EPC/Chol liposomal doxorubicin (kit) versus radiolabeled liposomes (tracer). Liposomes were injected i.v. into the lateral tail vein of female BDF1 mice at a drug dose of 20 mg/kg and a lipid dose of 73.5 mg/kg (drug to lipid ratio 0.27). At the indicated time points, mice (four per group) were sacrificed and plasma was assayed for lipid (**A**) and drug (**B**). Drug to lipid ratios are plotted in **C**. (● free doxorubicin, ■ tracer liposomes, ▲ EPC/Chol liposomes). Theoretical values of drug and lipid at t = 0 are ~40 µg doxorubicin/100 µl plasma and ~140 µg lipid/100 µl plasma based on 20-g mice. Data are means ±SD of four assays; where error bars are not visible, they are smaller than the size of the symbol. Asterisks indicate no significant difference between data points (*$P < 0.02$, **$P < 0.05$)

Preparation of cell suspensions from solid tumors

Solid tumors were excised and finely chopped in a culture dish with a razorblade. Tumor pieces were collected in a polypropylene tube and 5 ml PBS added. The tube was capped, shaken lightly and the tumor subjected to a modified trypsin digestion as outlined originally by Hill and Stanley [10]. Dispase (20 mg/ml) and collagenase (4 mg/ml) were prepared in PBS and 0.3 ml of each solution was added to the chopped tumor. Tumor tissues were then incubated in an oven, rotated at 37 °C for 45 min and 0.3 ml of DNase I (3 mg/ml) was added. The tumor solution was transferred to a new polypropylene tube through an 80-µm sterile filter and centrifuged at 500 *g* for 6 min. The resulting pellet was resuspended in 4 ml RPMI-1640 containing 15% FBS to generate

a single-cell suspension. This was diluted twice at a dilution of 1:9 in RPMI-1640. Cell counts and viability (trypan blue) were then determined using a hemocytometer.

Results

The plasma elimination characteristics of drug and liposomal lipid following i.v. administration of EPC/Chol liposomal doxorubicin are shown in Fig. 1. Liposomal lipid levels in the plasma indicated that 90, 77, and 23% of the injected liposomal lipid was retained in the blood compartment 1, 4, and 24 h after administration, respectively (Fig. 1A). Plasma levels of drug obtained after i.v. administration of doxorubicin in EPC/Chol liposomes were significantly greater than those achieved following administration of free drug (Fig. 1B, $P < 0.05$ at all time-points). In combination, the data shown in Fig. 1A and B allowed an estimation of drug retention for these liposomes (Fig. 1C), which indicated that 58% of the encapsulated drug was released from the circulating liposomes within 1 h of administration (consistent with previous reports [4, 20]). Doxorubicin elimination rates were essentially identical when the drug was encapsulated in the liposomes prepared with and without added tracer liposomes (Fig. 1B).

Drug and liposomal lipid biodistribution studies within a Lewis lung carcinoma tumor were initiated 10 days after tumor cell inoculation (Fig. 2). A time- and dose-dependent increase in liposomal lipid delivery to the established tumor was evident (Fig. 2A), demonstrating that liposome accumulation in extravascular sites was a slow process (as seen in other studies [5]). Drug accumulation in the tumor following administration of free and liposomal doxorubicin (Fig. 2B) did not appear to be time-dependent. Values for the AUC (area under the plasma concentration-time curve) following administration of liposomal drug at 20 and 40 mg/kg showed that total exposure within the tumor was increased 2.5-fold from an AUC of 870 to 2200 μg g^{-1} h, respectively. Drug levels measured at 4 h were comparable to levels measured at 72 h; however, a dose-dependent increase in drug levels was achieved as the dose of liposomal doxorubicin was increased from 20 to 40 mg/kg. Furthermore, the level of drug achieved within the tumor following injection of liposomal doxorubicin was two- to threefold greater than that achieved following administration of a comparable dose of free drug.

To assess the cellular association of liposomal doxorubicin with Lewis lung carcinoma cells, two fluorescent microscopy approaches were used. First, since doxorubicin is a fluorescent drug, fluorescent microscopy was used to evaluate its distribution using a 530–560 nm bandpass filter. Second, fluorescent microscopy was also used to identify MAC-1-positive cells (macrophages, monocytes, and neutrophils) with an FITC-labeled anti-MAC-1 (CD11b) antibody. The results (Fig. 3) are consistent with the data shown in Fig. 2 and show that drug delivery within the tumor was greater for the liposomal doxorubicin formulation when compared with free drug at the equivalent dose (20 mg/kg). In addition, doxorubicin fluorescence was

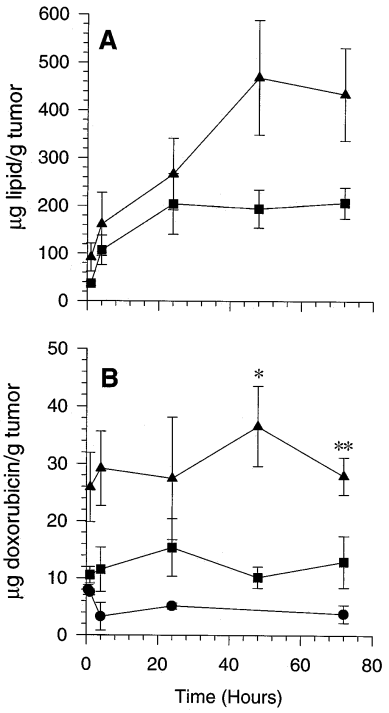

Fig. 2A, B Doxorubicin and lipid accumulation within the Lewis lung tumor. Female BDF1 mice (four per group) were injected with 3×10^5 Lewis lung cells subcutaneously in the flank. Ten days following tumor cell inoculation mice were treated i.v. with free doxorubicin (20 mg/kg), EPC/Chol liposomes (20 mg/kg), or EPC/Chol liposomes (40 mg/kg). Mice were sacrificed at the indicated time point following treatment and assayed for lipid (**A**) and drug (**B**) (● free doxorubicin, 20 mg/kg; ■ EPC/Chol liposomes, 20 mg/kg; ▲ EPC/Chol liposomes, 40 mg/kg). Data are means ± SD of three assays; where error bars are not visible, they are smaller than the size of the symbol. The doxorubicin levels following liposomal treatment at 20 mg/kg and 40 mg/kg were significiantly different ($*P < 0.007$, $**P < 0.02$)

frequently associated with MAC-1-positive cells (indicated by FITC-labeled anti-MAC-1 antibodies, some of which are indicated by arrows as examples). A small proportion of the doxorubicin, however, was associated with MAC-1-negative cells (areas of high doxorubicin fluorescence with no MAC-1 FITC fluorescence).

A second approach used to assess cell delivery to the Lewis lung tumor was based on the preparation of tumor-derived single-cell suspensions (see Methods). The cell suspensions typically had a viability of less than 50% and were devoid of MAC-1-positive cells (as assessed by flow cytometry and fluorescent microscopy). Loss of MAC-1-positive cells may have been a consequence of protease-mediated loss of the antigen from the isolated cells or, alternatively, may have been lost as a consequence of adherence to the plastic-ware used during the processing and washing of the isolated cells. To estimate drug levels in tumor cells, it can be assumed that a 1-g tumor consists of 10^9 cells [24]. On this basis, the data in Fig. 2B indicate that, for mice given the liposomal drug at a drug dose of 40 mg/kg, 150 ng of doxorubicin would be associated with 10^7 cells provided that 100% of the tumor-associated drug is cell-associated. If this value is related to a concentration,

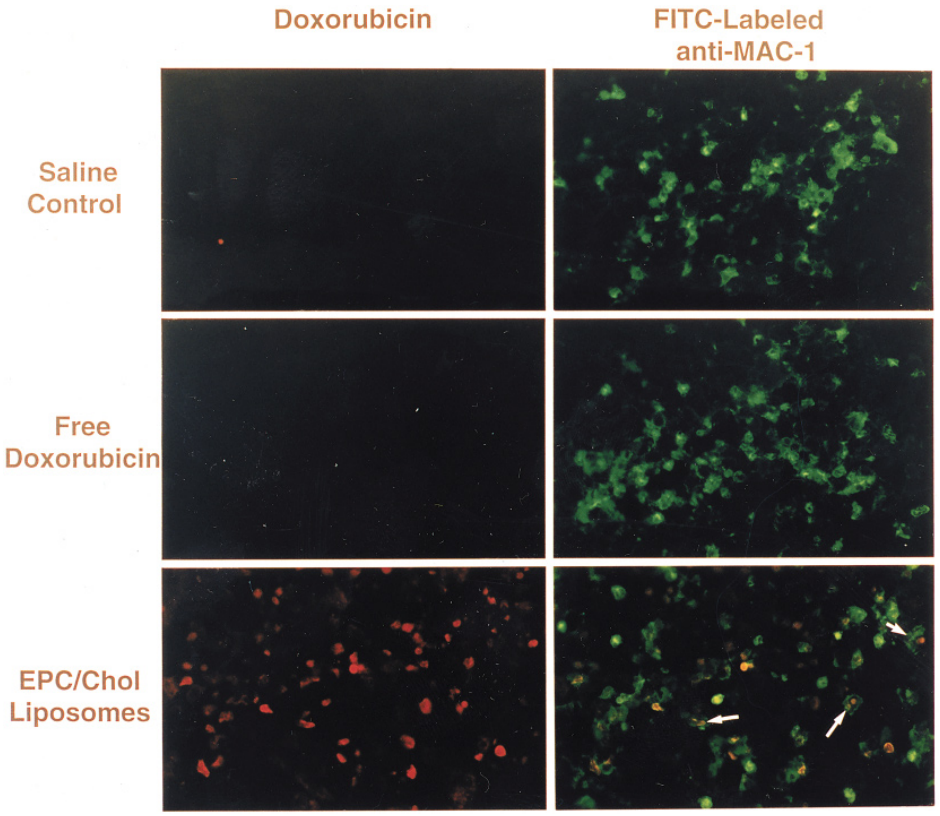

Fig. 3 Fluorescent microscopy of Lewis lung tumors from BDF1 mice treated with free doxorubicin and EPC/Chol liposomal doxorubicin. Female mice (three/group) were injected subcutaneously with 3×10^5 Lewis lung cells. Mice were treated i.v. 10 days following tumor cell inoculation with a saline control, free doxorubicin (20 mg/kg) or EPC/Chol liposomal doxorubicin (20 mg/kg). Mice were sacrificed 1 h after treatment and isolated tumors prepared for microscopy. Macrophages were identified using an FITC-labeled anti-MAC 1 antibody and doxorubicin-containing cells were identified using a rhodamine filter. *Arrows* indicate some of the cells positive for doxorubicin and MAC-1 as examples

assuming a 1-g tumor approximates 1 ml, then the liposomes were delivering ~15000 ng drug/ml. For Lewis lung cells in culture IC_{90} values for doxorubicin are ~500 ng/ml (<1000 n*M*), therefore, we would anticipate that there is sufficient drug present to exert cytotoxic effects. Following the preparation of the single-cell suspension, however, the level of cell-associated doxorubicin (assay of 800 µl of 10^7 cells/ml) was below the detection limits (1 ng/800 µl) of the assay. There was no measurable level of liposomal lipid found in this isolated cell population.

A similar analysis of doxorubicin and liposomal lipid accumulation in an established murine B16/BL6 solid tumor was also completed. The results, shown in Fig. 4, are based on a single time-point, 24 h. As anticipated, both liposomal doxorubicin (Fig. 4A) and lipid (Fig. 4B) accumulated in this tumor. When the tumor levels of free and liposomal drug were compared, following administration of equivalent drug doses, 40% more drug was obtained in tumors from mice treated with the liposomal formulation. At the maximum tolerated dose (MTD, 40 mg/kg) of EPC/Chol liposomal doxorubicin, there was a greater than twofold increase in tumor-associated drug compared with free drug-treated mice. This is in contrast to the tenfold increase observed using the Lewis lung carcinoma model. As noted here, doxorubicin delivery following administration of the liposomal drug is associated with accumulation of liposomal lipid (Fig. 4B). Cell suspensions prepared through a nonenzymatic approach (mincing the tumor with scissors prior to filtration through 80-µm filters) were also evaluated with the B16/BL6 tumors. As with the Lewis lung tumors, the resulting cells were washed, resuspended at a concentration of 10^7 cells/ml, and the level of cell-associated doxorubicin and lipid was measured. The drug and liposomal lipid levels associated with the isolated cells were below assay detection limits.

A comparative study of the extravasation phenomenon seen with liposomes in mice bearing the murine ascitic L1210 tumor is illustrated in Fig. 5. Results show that cells residing in the peritoneal cavity were exposed to five to ten times more drug at 24 h when doxorubicin was administered in liposomal form (Fig. 5A). Cell-associated doxorubicin accounted for approximately 50% of the drug measured in the peritoneal cavity of mice given the liposomal formulation. Importantly, following administration of free doxorubicin, 100% of the drug was localized within the peritoneal cells, a result that is consistent with previous studies [4]. Within the limitations of the two doses of liposomal doxorubicin studied (20 and 40 mg/kg) there appears to be a dose-dependent increase in doxorubicin delivery to cells residing in the peritoneal cavity. The results shown in Fig. 5B suggest that doxorubicin delivery to the peritoneal cavity was mediated, in part, by the liposomal carrier. An estimation of the drug-to-lipid ratio (wt/wt) in the isolated peritoneal cells gave a value of $0.09-0.11$ which was comparable to that measured in liposomes within the plasma compartment at this time-point (see Fig. 1C).

The level of macrophage-associated drug, measured using a procedure in which functional peritoneal macrophages were removed by adherence to plastic in culture, is shown in Fig. 6. It should be noted that in the absence of an ascitic tumor $4-6\times10^6$ peritoneal cells can be recovered

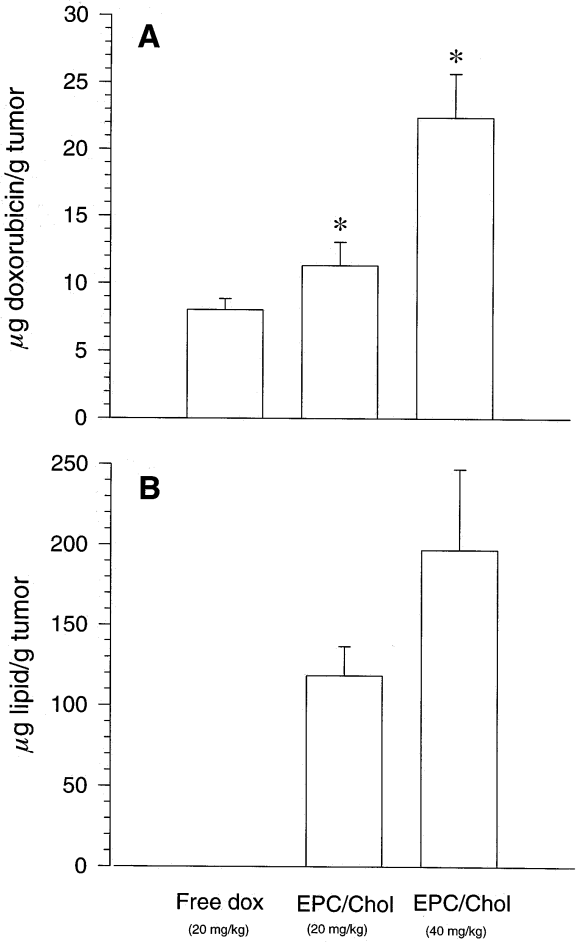

Fig. 4A, B Levels of B16/BL6 tumor-associated liposomal lipid and doxorubicin following administration of free drug and EPC/Chol liposomal doxorubicin. Female BDF1 mice (four per group) were injected with 2×10^5 B16/BL6 cells subcutaneously in the flank. Mice were treated i.v. 2 weeks following tumor cell inoculation, with free doxorubicin or the indicated dose of EPC/Chol liposomal doxorubicin. Mice were sacrificed 24 h following treatment and levels of drug (**A**) and lipid (**B**) were assayed. Data points are the means of 12 assays from three experiments ±SD. *Asterisks* indicate a significant difference from free drug ($*P<0.01$)

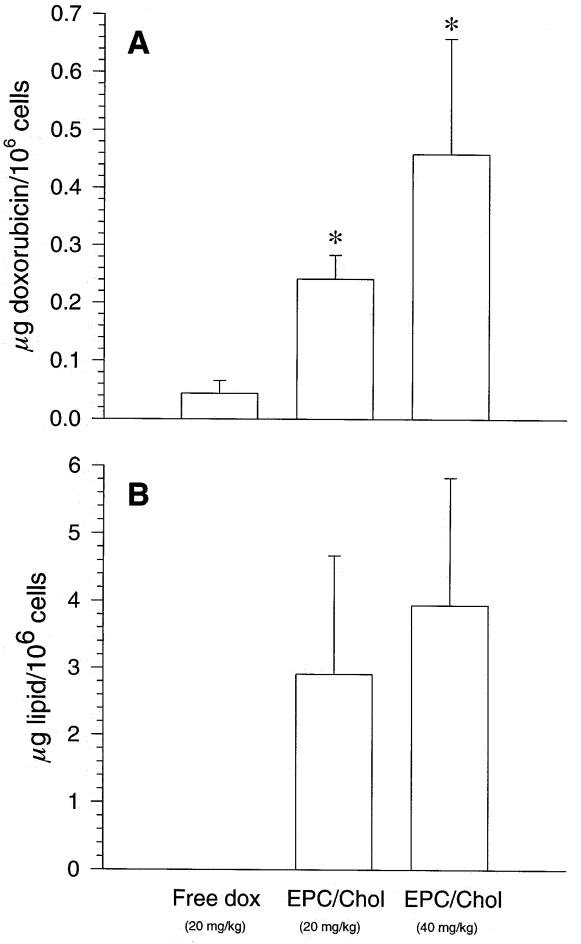

Fig. 5A, B Accumulation of EPC/Chol liposomal doxorubicin and free doxorubicin in the peritoneal cavity of L1210 tumor-bearing mice 24 h after i.v. administration. Female BDF1 mice (four per group) were injected i.p. with 1×10^5 L1210 cells, and 24 h later were treated either with free drug or EPC/Chol liposomal doxorubicin at the indicated doses. Mice were sacrificed 24 h after treatment and isolated peritoneal cells were assayed for drug (**A**) and lipid (**B**). Data are means ±SD of four assays. *Asterisks* indicate a significant difference from free drug ($*P<0.01$)

following peritoneal lavage. Typically, 30–40% of these cells will be easily distinguished by flow cytometry techniques as macrophages on the basis of forward and side light scattering characteristics. In the presence of tumors, however, the number of cells isolated by peritoneal lavage increases to more than 2×10^7. We have determined that cultured L1210 cells cannot be distinguished from peritoneal macrophages on the basis of size and granularity characteristics. Our approach, therefore, involved evaluating the level of macrophage-associated drug using a procedure in which functional peritoneal macrophages were removed by adherence to plastic in culture. For cells isolated from control mice, more than 95% of the cell-associated lipid was removed by the adherence step (Fig. 6A). In addition, a 70% reduction in cell-associated doxorubicin was observed compared with cells not depleted of macrophages (Fig. 6B). When adherent cells were removed from peritoneal cells isolated from L1210 tumor-bearing mice there was an 81% and 76% reduction in cell-associated liposomal lipid and doxorubicin, respectively. These results demonstrate that the majority of cell-associated doxorubicin in this ascitic tumor resides in adherent cells which are probably resident and/or induced macrophages.

Discussion

The studies summarized have been designed to characterize the target site distribution of an EPC/Chol liposomal doxorubicin formulation. Several reported studies have shown that liposomes administered i.v. exhibit a time-dependent movement from the blood compartment to ex-

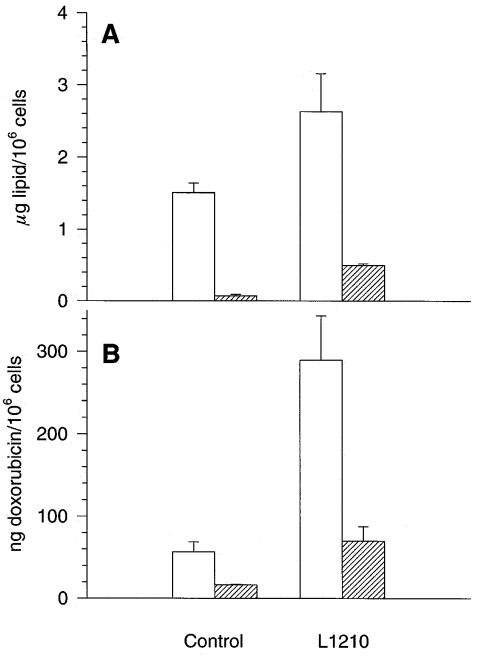

Fig. 6A, B Drug and lipid association to adherent peritoneal cells before and after adherent cell removal. Control (no tumor) and L1210 tumor-bearing mice (four per group) were injected i.v. with EPC/Chol liposomal doxorubicin (20 mg/kg) 24 h prior to isolation of peritoneal cells. Adherent cells were removed from peritoneal cells via a 2-h incubation in a culture flask. The association of lipid (**A**) and drug (**B**) to the adherent and nonadherent cells was determined as outlined in Materials and methods. (*Open bars* before adherent cell removal, *hatched bars* after adherent cell removal). Data are means ±SD of four assays

travascular sites [2, 7, 8, 22] and in particular the peritoneal cavity [5]. Although these studies show that drug delivery to sites of tumor growth can be enhanced significantly through the use of liposomal drug carriers, it has yet to be established (preclinically or clinically) whether controlled drug release and/or use of factors that enhance tumor delivery would lead to enhanced therapeutic activity. There is little evidence supporting the concept that the therapeutically active agent is liposome-associated doxorubicin. In contrast, it is likely that drug released from liposomes, in the circulation, in the interstitial space of progressing tumors, or from liposomes that have been degraded within the lysosomal compartment of phagocytic cells [23], is the biologically active agent. The studies reported here were developed to gain a better understanding of the mechanism of action of an EPC/Chol liposomal anticancer drug. Two factors of interest are discussed, drug release and the intracellular distribution of liposome-associated drug following extravasation in regions of tumor growth.

It can be suggested that for both ascitic and solid tumor models, improved therapy may be a consequence of enhanced drug exposure at the site of tumor growth achieved through the use of a liposomal carrier. The use of an ascitic tumor model for assessing potential therapeutic mechanisms of a liposomal anticancer drug as shown in Figs. 5 and 6 is simple but easily criticized as ascitic tumors do not structurally resemble solid tumors in several important ways. First, unlike solid tumors, ascitic tumors do not exhibit angiogenesis. Second, in contrast to solid tumors there is an absence of a defined matrix structure allowing the tumor cells and associated host-derived cells in the ascitic tumor to progress in the fluid environment. Third, macromolecules that have extravasated into the site will have an increased probability of direct interaction with the cell populations present. Therefore, it was not surprising that the levels of cell-associated drug (and liposomal lipid) were apparently greater when the ascitic model was used in comparison with either solid tumor. However, under these ideal conditions (for an in vivo model) by far the major amount of all drug delivered to the tumor is either non-cell-associated or bound in macrophages. Even vast differences in the pathology between the solid tumors showed no improvements in drug or lipid accumulation. The murine melanoma has a vasculature that is characterized by the presence of blood channels [27] and lacks a well-defined intratumor matrix. In contrast, the Lewis lung carcinoma is well vascularized and is known to produce significant quantities of VEGF (vascular endothelial cell growth factor).

Since it can be shown using tumors (not expressing a multidrug-resistant phenotype) that free doxorubicin efficiently accesses and is effectively retained by progressing tumors [6], improvements in tumor cell exposure to drug may best be achieved with liposomes that release their contents (EPC/Chol) at well-defined rates or under controlled conditions. For mice bearing Lewis lung carcinoma, the EPC/Chol liposomal doxorubicin formulation mediates an increase in tumor-associated drug levels; however, the levels obtained are independent of the time evaluated after administration. These increased levels may have been achieved by the liposomes acting as sustained-release systems for free drug, allowing more drug to accumulate in the tumor than from single doses of free drug, or by the accumulation of drug in the tumor as a combination of free drug uptake plus uptake of drug still retained in the liposomes. Either way, the drug accumulation profiles were contrary to what has been observed with saturated liposomal doxorubicin formulations which produce a time-dependent drug accumulation [5, 18, 19]. It should also be noted that the levels of drug achieved in the Lewis lung tumor following i.v. administration of the EPC/Chol liposomal formulation were less than those observed for saturated liposomal carrier systems. Since the EPC/Chol liposomes release their contents into the circulation over a reasonable time frame (70% drug loss over 24 h), free doxorubicin accumulation within the tumor can progress in the absence of efficient liposome accumulation. Clearly, identifying the role of controlled drug release from liposomes into the circulation prior to liposome-mediated drug accumulation in tumors is essential. It can be suggested that the steady-state drug levels observed are a consequence of using liposomes that exhibit poor drug retention characteristics. Importantly, peak free drug levels are avoided when drug is encapsulated in this liposomal carrier system which

contributes to the well-established reduction in cardiotoxicity for the EPC/Chol liposomal formulation [3, 9, 14].

Increased drug release, however, is incompatible with drug carrier systems designed to achieve maximum drug levels in the tumor, where drug retention and liposome circulation longevity are key optimization parameters. Although we and others have shown that liposomes can leave blood vessels and enter extravascular sites, the rate of extravasation is slow. This is primarily a consequence of molecular size [16, 21]. Liposomal anticancer agents displaying optimal activity typically exhibit a size distribution between 80 and 160 nm [18]. It is anticipated that such structures could only cross blood vessel walls that exhibit fenestrations or pores, although movement via transcytotic vesicles cannot be excluded [12]. Regardless of the mechanism of liposome extravasation, optimal drug delivery will be achieved with liposomes that retain drug effectively during the time period required for extravasation to occur. If liposomes are retained in the circulation for extended time periods then the probability for the carrier to extravasate will increase.

The results presented demonstrate that both liposomes and associated drug accumulate at sites of solid tumor growth. The extent of accumulation was dependent on tumor type and for liposomal lipid this accumulation process was time dependent. Since the liposomal carriers used in the study are known to release drug, the kinetics (see Fig. 2) of drug accumulation were different and the level of drug obtained was relatively constant over a 3-day time course. Even though there were significant drug and liposomal lipid levels in these tumors, analysis of single-cell suspensions derived from these tumors suggested that there was little cell-associated material.

Optimization of liposome drug retention and extravasation properties must proceed, together with the development of mechanisms for efficient release of encapsulated drug from regionally localized liposomes. An elegant example of such an approach, based on temperature-induced destabilization of the liposomal carrier, has recently been published [13, 26]. Alternatively, we are developing approaches that will promote binding and intracellular delivery of regionally localized liposomal drugs to specific cells within the site. These results suggest that it may be possible to achieve tumor cell specific-delivery. A significant proportion of regionally localized liposomal carriers reside within the tumor's interstitial space and, provided liposome movement is not restricted (i.e. the liposomes are not bound to matrix components), the target cell-specific delivery of the liposomes to tumor cells may be possible. For this reason, studies in this laboratory have now focused on maximizing the tendency for liposomes, with surface associated targeting ligands, to access regions of tumor growth. Subsequently, studies will evaluate the intratumor distribution of liposomal drugs exhibiting optimal binding characteristics with the objective of demonstrating target cell-specific delivery.

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References

1. Allen TM, Chonn A (1987) Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett 223:42
2. Bakker-Woudenberg IA, Lakerse AF, ten-Kate MT, Storm G (1992) Enhanced localization of liposomes with prolonged blood circulation time in infected lung tissue. Biochim Biophys Acta 1138:318
3. Balazsovits JA, Mayer LD, Bally MB, Cullis PR, McDonell M, Ginsberg RS, Falk RE (1989) Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin. Cancer Chemother Pharmacol 23:81
4. Bally MB, Nayar R, Masin D, Hope MJ, Cullis PR, Mayer LD (1990) Liposomes with entrapped doxorubicin exhibit extended blood residence times. Biochim Biophys Acta 1023:133
5. Bally MB, Masin D, Nayar R, Cullis PR, Mayer LD (1994) Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumor bearing mice. Cancer Chemother Pharmacol 34:137
6. Broxterman HJ, Feller N, Kuiper CM, Boven E, Versantvoort CH, Teerlink T, Pinedo HM, Lankelma J (1995) Correlation between functional and molecular analysis of mdr1 P-glycoprotein in human solid-tumor xenografts. Int J Cancer 61:880
7. Gabizon A (1992) Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res 52:891
8. Gabizon A, Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85:6949
9. Gill PS, Espina BM, Muggia F, Cabriales S, Tulpule A, Esplin JA, Liebman HA, Forssen E, Ross ME, Levine AM (1995) Phase I/II clinical and pharmacokinetic evaluation of liposomal daunorubicin. J Clin Oncol 13:996
10. Hill RP, Stanley JA (1975) The lung-colony assay: extension to the Lewis lung tumor and the B16 melanoma-radiosensitivity of B16 melanoma cells. Int J Radiat Biol 27:377
11. Hope MJ, Bally MB, Webb G, Cullis PR (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim Biophys Acta 812:55
12. Huang SK, Martin FJ, Jay G, Vogel J, Papahadjopoulos D, Friend DS (1993) Extravasation and transcytosis of liposomes in Kaposi's sarcoma-like dermal lesions of transgenic mice bearing the HIV tat gene. Am J Pathol 143:10
13. Huang SK, Stauffer PR, Hong K, Guo JW, Phillips TL, Huang A Papahadjopoulos D (1994) Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. Cancer Res 54:2186
14. Kanter PM, Bullard GA, Ginsberg RA, Pilkiewicz FG, Mayer LD, Cullis PR, Pavelic ZP (1993) Comparison of the cardiac effects of liposomal doxorubicin (TLC D-99) versus free doxorubicin in beagle dogs. In Vivo 7:17
15. Kanter PM, Bullard GA, Pilkiewicz FG, Mayer LD, Cullis PR, Pavelic ZP (1993) Preclinical toxicology study of liposome encapsulated doxorubicin (TLC D-99): comparison with doxorubicin and empty liposomes in mice and dogs. In Vivo 7:85
16. Litzinger DC, Buiting AM, van Rooijen N, Huang L (1994) Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. Biochim Biophys Acta 1190:99
17. Mayer LD, Hope MJ, Cullis PR, Janoff AS (1986) Solute distribution and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim Biophys Acta 817:193

18. Mayer LD, Tai LCL, Ko DSC, Masin D, Ginsberg RS, Cullis PR, Bally MB (1989) Influence of vesicle size, lipid composition and drug to lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res 49:5922
19. Mayer LD, Bally MB, Cullis PR, Wilson SL, Emerman JT (1990) Comparison of free and liposomal encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor. Cancer Lett 53:183
20. Mayer LD, Cullis PR, Bally MB (1994) The use of transmembrane pH gradient-driven drug encapsulation in the pharmacodynamic evaluation of liposomal doxorubicin. J Liposome Res 4:529
21. Nagy JA, Herzberg KT, Masse EM, Zientara GP, Dvorak HF (1989) Exchange of macromolecules between plasma and peritoneal cavity in ascites tumor-bearing, normal, and serotonin-injected mice. Cancer Res 49:5448
22. O'Sullivan MM, Powell N, French AP, Williams KE, Morgan JR, Williams BD (1988) Inflammatory joint disease: a comparison of liposome scanning, bone scanning and radiography. Ann Rheum Dis 47:485
23. Storm G, Steerenber PA, Emmen F, van Borssum WM, Crommelin DJ (1988) Release of doxorubicin from peritoneal macrophages exposed in vivo to doxorubicin-containing liposomes. Biochim Biophys Acta 965:136
24. Tannock IF (1989) Principles of cell proliferation. Cell kinetics. In: Devita VT, Hellman S, Rosenberg SA (eds) Cancer. Principle and practice of Oncology, 3rd edn. J.B. Lippincott, Philadelphia, p 3
25. Thies RL, Cowens DW, Cullis PR, Bally MB, Mayer LD (1990) Method for rapid separation of liposome-associated doxorubicin from free doxorubicin in plasma. Anal Biochem 188:65
26. Unezaki S, Maruyama K, Takahashi N, Koyama M, Yuda T, Suginaka A, Iwatsuru M (1994) Enhanced delivery and antitumor activity of doxorubicin using long-circulating thermosensitive liposomes containing amphipathic polyethylene glycol in combination with local hyperthermia. Pharm Res 11:1180
27. Warren BA (1979) The vascular morphology of tumors. In: Hans-Inge Peterson (ed) Tumor blood circulation. Angiogenesis, vascular morphology and blood flow of experimental and human tumors. CRC Press, Boca Raton, p 1
28. Wu NZ, Da D, Rudoll TL, Needham D, Whorton AR, Dewhirst MW (1993) Increased microvascular permeability contributes to preferential accumulation of stealth liposomes in tumor tissue. Cancer Res 53:3765