Cancer Chemother Pharmacol (1995) 36: 91–101

ORIGINAL ARTICLE

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A two-step targeting approach for delivery of doxorubicin-loaded liposomes to tumour cells in vivo

Received: 21 March 1994 / Accepted: 22 September 1994

Abstract A two-step targeting approach was used to deliver doxorubicin-loaded liposomes to a murine tumour cell (P388 leukaemia) grown in culture and, more importantly, in vivo. Targeting was mediated through the use of an antibody specific for the Thy 1.2 antigen that is highly expressed on P388 cells. Briefly, the approach consists of prelabeling target cells with biotinylated anti-Thy 1.2 antibody prior to administration of drug-loaded liposomes that have streptavidin covalently attached to their surface. Results from in vitro studies demonstrate that a 30-fold increase in cell-associated lipid and a 20-fold increase in cell-associated doxorubicin can be achieved over control liposomes using this two-step procedure. Flow-cytometry and fluorescent-microscopy data were used to confirm that P388 cells can be stably labeled with the biotinylated anti-Thy 1.2 antibody in vivo. Subsequently, liposome-targeting studies were initiated in vivo, where target cell binding was assessed following i.p. or i.v. injection of doxorubicin-loaded liposomes into animals bearing P388 tumours prelabeled with biotinylated antibody. A streptavidin-mediated 3.7-fold increase in cell-associated lipid and drug was achieved when the liposomes were given i.p. When doxorubicin-loaded streptavidin liposomes were injected i.v., P388 cells located in the peritoneal cavity were specifically labeled, although the efficiency of this targeting reaction was low. Less than a 2-fold increase in cell-associated lipid was achieved through the use of target-specific (streptavidin-coated) liposomes. These studies demonstrate that the presence of a well-labeled target cell population within the peritoneal cavity will not promote accumulation of an i.v. injected, targeted liposomal drug. Furthermore, the importance of separating target-cell-specific binding from non-specific uptake by tumour-associated macrophages is discussed.

Key words Liposomes · Targeting · Doxorubicin

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Introduction

The therapeutic index of certain drugs can be improved, sometimes dramatically, when they are given in association with a liposomal carrier. This has been best characterised for the anticancer drug doxorubicin, where it has been shown that liposomally encapsulated drug is less toxic and more efficacious than free drug [5, 13–15, 19, 27, 30, 33, 36]. Reduced toxicity and enhanced anti-tumour activity are thought to be a consequence of liposome-mediated changes in pharmacokinetics and biodistribution. For example, the chronic dose-limiting toxicity of doxorubicin is cardiotoxicity, a toxicity that is reduced significantly when the drug is given in liposomes [5, 13, 15, 30, 36]. Since liposomes do not accumulate in cardiac tissue, this reduction may be attributed to a decreased availability of free drug in this tissue. Conversely, increased anti-tumour activity can be attributed to the tendency of (small) liposomes to accumulate to significant levels at sites of tumour growth [31].

A significant advance in the development of liposomal anticancer agents has come from the use of liposomes containing G_{M1} or poly(ethylene glycol)-modified phosphatidylethanolamine (PEG-PE) that exhibit long circulation lifetimes [3, 18, 31]. Importantly, it has been demonstrated that such liposomes readily access sites of tumour growth. In terms of achieving cell-specific targeting, this passive tendency to accumulate in sites of tumour growth is a fundamental requirement for targeting of liposomes. One possible limitation of antibody-coated liposomes is that they are immunologically better recognised than protein-free liposomes and, consequently, exhibit shortened circulation lifetimes following i.v. administration [11]. Elimina-

tion of i.v. injected antibody-coated liposomes may be due to Fc-mediated clearance [4] or to the observation that protein-liposome coupling technology promotes the aggregation of liposomes [24, 25]. Liposome aggregates will exhibit reduced circulation lifetimes due to their increased size [24, 35]. Regardless of the mechanisms involved, a reduction in circulation longevity will reduce the tendency of these protein-coated liposomes to access a target-cell population residing in an extravascular site.

We have developed an alternative approach to achieve targeting in vivo that specifically addresses issues regarding in vivo circulation and targeted liposome extravasation. The targeting procedure relies on the use of streptavidin-coated liposomes (SA-LUVs) that bind specifically to target cells prelabeled with a biotinylated antibody [23, 24]. These liposomes have been used to label defined cell populations in vitro and exhibit in vivo circulation lifetimes comparable with those of control liposomes that lack surface-associated protein. The studies presented herein extend these previous investigations by characterising SA-LUV targeting to murine lymphocytic leukaemia cells (P388) in vivo. Targeting is achieved by prelabeling P388 cells with biotinylated anti-Thy 1.2 antibody. It is shown that this two-step targeting approach can target P388 cells (residing in the peritoneal cavity), although the efficiency of targeting is reduced significantly as compared with that achieved in vitro.

Materials and methods

Materials

Distearoyl phosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids, and *N*-succinimidyl 3-(2-pyridyldithio) propionic acid (SPDP) was supplied by Molecular Probes. Doxorubicin was obtained from Adria Laboratories of Canada (Mississauga, Ontario), and biotinylated anti-Thy 1.2 antibody and normal mouse serum were purchased from Cedarlane Laboratories (Hornby, Ontario). Cholesterol, dithiothreitol (DTT), 2,2′-azino-di-(3-ethylbenzthiazdine suphonate) (ABTS), Triton-X-100, β-mercaptoethanol, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES), *N*-ethylmaleimide (NEM), bovine serum albumin (BSA), streptavidin (SA), Sephadex G-50, D-biotin, sepharose CL-4B, and all salts were obtained from Sigma. [^{3}H]-Cholesteryl hexadecyl ether was obtained from NEN, and [^{14}C]-biotin was supplied by Amersham. Purified *N*-[4-(*P*-maleimidophenyl)butyzyl]-dipalmitoyl phosphatidylethanolamine (MPB-DPPE) was purchased from Northern Lipids Inc. (Vancouver, Canada).

Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared using the extrusion method described by Hope et al. [17]. Briefly, lipid films (54 mol% DSPC, 45 mol% cholesterol, 1 mol% MPB-PE) were prepared from a chloroform solution by drying under a stream of nitrogen followed by vacuum evaporation for 2 h. Lipids were then hydrated at 65 °C in 300 m*M* citrate (pH 4.0) by vortex mixing such that a final lipid concentration of 50 m*M* was achieved. LUVs were then prepared by extrusion (five times) at 65 °C through stacked polycarbonate filters (100 nm pore size; Nucleopore, Inc.) employing an extruder (Lipex Biomembranes, Vancouver). LUVs were subjected to five freeze-thaw cycles followed by repeated extrusion (five times). Mean vesicle diameters were determined by quasielastic light scattering (QELS) using a Nicomp 270 submicron particle sizer operating at a wavelength of 632.8 nm. The exterior pH was raised for the coupling reaction by passing the vesicles (pH 4.0) down a Sephadex G-50 (medium) column (1.5 × 20 cm) pre-equilibrated with 150 m*M* NaCl and 25 m*M* HEPES (pH 7.5, HBS).

SPDP modification of streptavidin

Streptavidin (5 mg/ml in HBS) was modified with the amine-reactive agent SPDP according to published procedures [23]. Briefly, SPDP (25 m*M* in methanol) was incubated at a 10-fold molar ratio to streptavidin at room temperature for 10 min. SPDP-modified streptavidin was reduced with dithiothreitol (DTT; 10-fold molar excess over SPDP, 10 min) and passed down a Sephadex G-50 column equilibrated with HBS to remove unreacted SPDP and DTT. Reduced SPDP-modified streptavidin was immediately used for coupling experiments. The extent of modification of streptavidin was determined by estimating both the concentration of the protein at 280 nm (molar extinction coefficient (E_{280}), 1.66×10^5) prior to the addition of DTT and the 2-thiopyridine concentration at 343 nm (E_{343}, 7550) at 10 min after the addition of DTT according to published procedures [10].

Coupling of SPDP-streptavidin to liposomes

The coupling of SPDP-modified streptavidin to liposomes was performed in a modified version of the method used by Loughrey et al. [23]. Briefly, SPDP-streptavidin was incubated with liposomes at a ratio of 75 μg protein/μmol lipid (10 m*M* final lipid concentration). The coupling reaction was stopped after 12 min by the addition of NEM (100-fold molar excess over SPDP), and unassociated protein was removed by gel filtration on Sepharose CL-4B equilibrated with 300 m*M* citrate (pH 4.0). Cross-linking of vesicles during coupling had resulted in liposome aggregation (300–400 nm in diameter as determined by QELS). Aggregated protein-vesicle conjugates were re-extruded through stacked polycarbonate filters (100 nm pore size) to generate a defined-size population (130–170 nm in diameter) as described elsewhere [24, 25]. The vesicle interior (300 m*M* citrate, pH 4.0) was maintained during re-extrusion as indicated above by passing the liposomes down a column equilibrated with citrate buffer. A transmembrane pH gradient was established by passing the re-extruded streptavidin-coated vesicles down a Sephadex G-50 column pre-equilibrated with HBS (pH 7.5). The extent of streptavidin coupling to liposomes was determined using a functional assay that measured binding of [^{14}C]-biotin to streptavidin. Briefly, SA-LUVs (0.50 μmol lipid in 0.5 ml) were incubated with [^{14}C]-biotin (7.31 nmol added, 46.9 nmol/μCi) for 10 min and the unbound biotin was removed by gel filtration on Sephadex G-50 (medium) equilibrated with HBS. The extent of [^{14}C]-biotin binding to SPDP-modified streptavidin obtained after gel filtration was used as a standard to calculate protein-to-lipid ratios.

Doxorubicin uptake into streptavidin-coated vesicles

Streptavidin liposomes (5–10 m*M* lipid) exhibiting a transmembrane pH gradient (interior acidic) were incubated with doxorubicin at a drug-to-lipid ratio of 0.2:1 (mol:mol) in HBS at 65 °C for 10 min. Free doxorubicin was separated from doxorubicin entrapped in SA-LUVs by column chromatography using Sephadex G-50 pre-equilibrated with HBS. In vitro drug retention was evaluated by placing 2 m*M* lipid in dialysis tubing (6–8,000 mwco, Spectrum Medical Industries, Inc., Los Angeles), 10% fetal calf serum (Gibco Labs) and dialysing against 25 ml 10% fetal calf serum at 37 °C. In vivo drug retention was evaluated by injection of drug-loaded liposomes via the lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg lipid/kg). Blood was collected via cardiac puncture and placed in ethylenediaminetetraacetic acid (EDTA)-treated microtainers (Becton-Dickinson, Canada). Plasma was prepared by centrifuging (200 *g*) blood samples for 10 min in a clinical centrifuge, and the lipid and doxorubicin content was quantified as described below.

Biotinylated anti-Thy 1.2 antibody binding to P388 cells

Murine leukaemia P388 cells grown in culture at 37 °C in an atmosphere containing 5% CO_2) were aliquoted (2×10^7 cells/ml) into conical bottomed polypropylene tubes and incubated with biotinylated anti-Thy 1.2 antibody (0–3.125 n*M*) in phosphate-buffered saline (PBS) containing 2% BSA (w/v) for 30 min at 4 °C. Cells were washed three times with PBS and then incubated with streptavidin peroxidase (10 μg/ml) for 30 min at 4 °C. After being washed three times with PBS, cells were assayed for peroxidase activity according to the procedure of Porstmann et al. [32]. Briefly, 100 μl of freshly prepared ABTS solution (30 μg ABTS in 0.2 *M* glycine buffer, pH 6.0, and 0.2% hydrogen peroxide) was added to cells (10^5) in 400 μl of acetate buffer (0.1 *M*, pH 4.0). The reaction was quenched at 1 h by the addition of 10 m*M* sodium azide. Cells were pelleted by centrifugation and supernatants were read at 410 nm on a Bausch and Lomb spectronic 2000 spectrophotometer.

In vitro targeting of streptavidin-coated liposomes to P388 cells

The murine lymphocytic leukaemia cell line P388 was obtained from NCI Tumour Repository and grown in RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum (FCS, Flow Laboratories). Murine leukaemia P388 cells grown in culture (at 37 °C in an atmosphere containing 5% CO_2) were aliquoted (10^7 cells/ml) into conical bottomed polypropylene tubes and incubated either with biotinylated anti-Thy 1.2 antibody or alone in PBS containing 2% BSA (w/v) for 30 min at 4 °C. The final antibody concentration for these studies was 12.5 n*M*. After being washed twice with PBS, cells were incubated with doxorubicin-loaded streptavidin-coated liposomes (2 m*M* lipid) for 30 min at 4 °C. The cells were then washed three times with PBS and the cell-associated lipid and doxorubicin content was assayed. Cells were also analysed by flow cytometry and fluorescent microscopy. Controls included cells incubated with doxorubicin-loaded liposomes not conjugated with streptavidin.

Peritoneal cavity lavage

Female BDF1 mice (18–22 g) were obtained from Charles River (Canada). Groups of four mice per experimental point were given the specified treatment in either an i.p. dose (500 μl volume) or an i.v. dose (200 μl volume) via the lateral tail vein. Peritoneal cavities were lavaged with 5 ml of indicator-free and Ca^{2+}/Mg^{2+}-free Hanks' buffered saline solution (HBSS), which was injected i.p. The abdomen was gently massaged and the peritoneal fluid was removed with a syringe equipped with a 22-gauge needle. Peritoneal fluid was assayed for liposomal lipid as indicated below. Mice bearing P388 tumours were prepared by inoculating mice i.p. with 1×10^6 P388 cells. Cell growth was allowed to progress for 4 days prior to i.p. injection of Ab or i.v. injection of specified liposomal preparation. At 4 days after cell administration there was no measurable ascites fluid, and lavage fluid that contained red cell contamination was discarded.

Quantitation of lipid and doxorubicin

Liposomal lipid was measured by incorporation of tracer quantities of the non-exchangeable, non-metabolizable radiolabeled lipid marker [^{3}H]-cholesteryl hexadecyl ether [7]. The amount of cell-associated lipid was then determined by liquid scintillation counting. Samples in PBS were mixed with 5 ml Pico-Fluor 40 scintillation cocktail (Packard, Canada) prior to counting on a Packard 1900 TR scintillation counter.

Cell-associated doxorubicin was measured using a fluorescent assay procedure as described by Bally et al. [7]. Briefly, samples to be assayed were diluted to 800 μl in dH_2O. Subsequently, 100 μl 10% sodium dodecyl sulphate (SDS) and 100 μl 10 m*M* H_2SO_4 were added prior to the addition of 2 ml isopropanol:chloroform (1:1, v/v). This two-phase system was vigorously mixed and frozen at −20 °C to promote protein precipitation. After thawing, the samples were centrifuged (500 *g* for 5 min) and the organic phase was carefully removed. Doxorubicin in the organic phase was measured by fluorescence (excitation wavelength, 500 nm; emission wavelength, 550 nm) using a Perkin-Elmer LS50-B spectrofluorometer. A standard doxorubicin curve was prepared using a similar extraction procedure. Differences in liposomal lipid and doxorubicin binding to cells were assessed using analysis of variance (ANOVA).

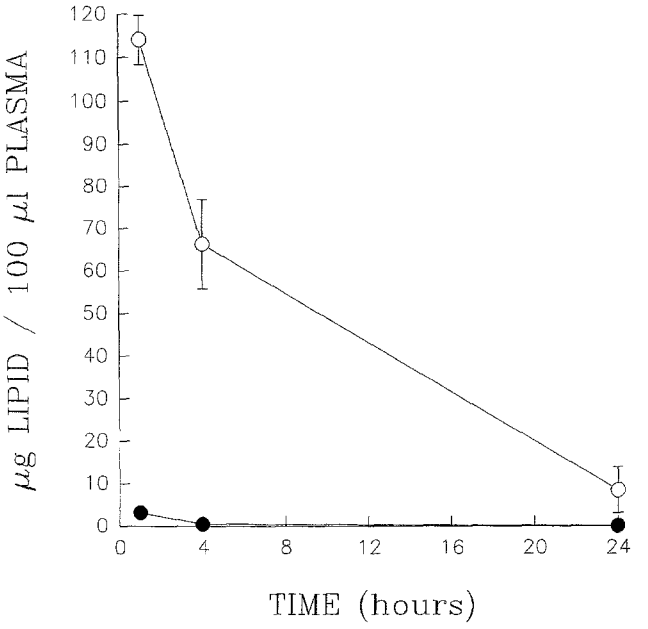

Fig. 1 Plasma clearance of streptavidin-coated and antibody-coated liposomes in tumour-free BDF1 mice. Streptavidin-coated liposomes (○) and antibody-coated liposomes (●) were injected via the lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg lipid/kg). The mice were killed at the indicated times and the level of lipsomal lipid was determined (see Materials and methods). The data shown represent mean values for at least 4 animals ±SEM

Flow-cytometric analysis

Cell-associated doxorubicin fluorescence was measured on an EPICS 753 flow cytometer (Coulter) with an excitation wavelength of 488 nm at 200 mW power with an argon ion laser. A 488-nm dichroic mirror and a 488-nm bandpass filter were used to measure the side-scatter signal. A 515-nm interference filter and a 488-nm laser blocking filter were used to block the excitation light from the fluorescence detectors. A 515-nm longpass filter was used to measure doxorubicin. Gates were set around the P388 cells using forward-scatter and side-scatter signals to exclude cell clumps and debris.

Fluorescent microscopy

Living cells were viewed with a Leitz Dialux fluorescence microscope with phase objectives and epifluorescence illumination. Original photographs were made with Fuji 400 colour slide film, with the exposure time being determined automatically by a photometer in the camera.

Cytotoxicity assays

Typically, P388 cells (1.0×10^7/ml) were first incubated with biotinylated anti-Thy 1.2 antibody (12.5 nM) for 30 min at 4 °C and then washed twice in RPMI media 1640 with 10% (v/v) FBS. Next, 10^6 cells were aliquoted into 24-well tissue-culture plates, exposed to

Table 1 Doxorubicin loading and retention in liposomes and streptavidin-coated liposomes

Liposome	Drug uptake[a]		Drug retention (drug to lipid ratio; mol/mol)	
	Rate (min)	Trapping efficiency (%)	In vitro[b]	In vivo[c]
LUV	<5	98	0.203	0.200
SA-LUV	<5	98	0.201	0.203

[a] Doxorubicin uptake, trapping efficiency and retentions were determined as described in Materials and methods

[b] Drug retention of doxorubicin-loaded liposomes was evaluated by dialysing 2 m*M* lipid in 10% fetal calf serum against 25 ml fetal calf serum at 37 °C for 4 h

[c] Drug retention was evaluated by injection of drug-loaded liposomes via the lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg lipid/kg). Blood was collected at 4 h via cardiac puncture and placed in EDTA-treated microtainers. Plasma was prepared by centrifuging (200 *g*) blood samples for 10 min in a clinical centrifuge, and the lipid and doxorubicin content was quantified as described in Materials and methods

either free drug or liposomal drug and incubated (37 °C, 5% CO_2) for 24 h at the indicated time, and an aliquot of each culture was counted on a Coulter Counter ZM to determine the total cell count. Viability was determined using the flow cytometric method of Ross et al. [34]. Briefly, propidium iodide (20 μg/ml final concentration) was incubated with cells for 30 min, after which fluorescence was assessed on an EPICS 753 flow cytometer. An argon laser at 488 nm was used for excitation of red fluorescence greater than 630 nm. The relative percentage of viability was determined by multiplying the percentage of viability by the total cell count for each culture.

Results

Previous reports from this laboratory have demonstrated that liposomes with covalently or non-covalently attached streptavidin can be targeted to specific cells via biotinylated antibodies [23]. Targeting can be achieved in two ways. First, biotinylated antibodies can be bound to streptavidin liposomes, forming an immunoliposome that can then be targeted to a selected cell population [22, 25]. A similar procedure has been used successfully by Ahmad et al. [1, 2] to prepare immunoliposomes with specificity to lung tumour cells in vitro and in vivo. Target cells can also be prelabeled with biotinylated antibody prior to addition of the streptavidin liposomes [23, 25]. This two-step approach was further developed in the present study for delivery of liposomes containing doxorubicin to murine P388 cells in vivo. One reason for pursuing a two-step approach addresses the observation that antibody-coated liposomes can be cleared from the circulation much faster than streptavidin-coated liposomes. This is illustrated for the murine model system employed herein by the data in Fig. 1. This comparison of the circulation lifetimes of SA-LUVs and SA-LUVs with bound biotinylated antibody (mAb-SA-LUVs) shows that the antibody-coated liposomes are cleared rapidly after administration. 1 h after i.v. injection, 52% of the injected dose of SA-LUVs was found in the circulation as compared with the 1.5% obtained after

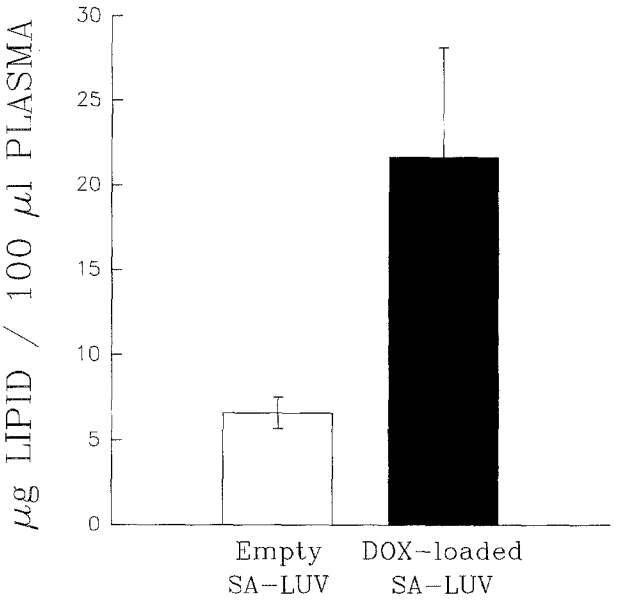

Fig. 2 Effect of encapsulated doxorubicin on the circulation lifetime of streptavidin liposomes. Doxorubicin-loaded (*solid bar*) or empty (*empty bar*) SA-LUVs were injected via the lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg lipid/kg). The mice were killed 24 h after liposome injection and the level of lipsomal lipid was determined (see Materials and methods). The data shown represent mean values for at least 4 animals ±SEM

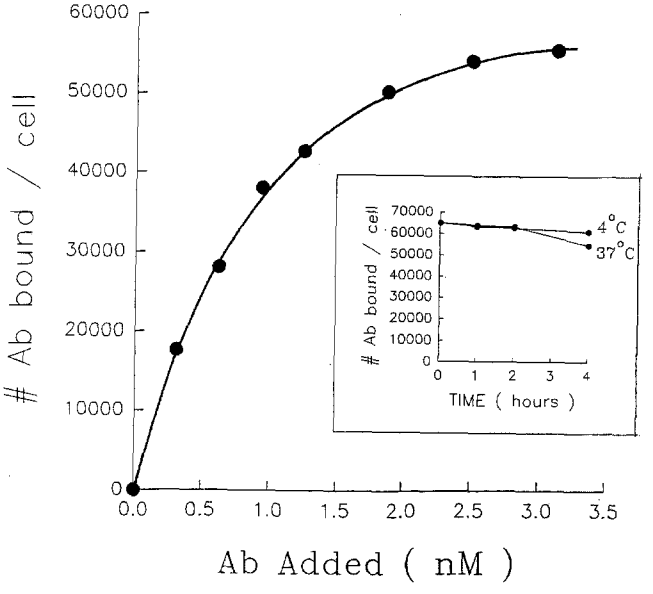

Fig. 3 Biotinylated anti-Thy 1.2 antibody binding to P388 cells. Murine leukaemia P388 cells (2×10^7) were incubated with biotinylated anti-Thy 1.2 antibody at the indicated concentrations. Bound antibody was determined by a colorimetric assay using streptavidin-peroxidase and the substrate ABTS, which was read at 410 nm, as described in Materials and methods. The level of antibody bound to the cell surface was monitored over time at 4 ° and 37 °C (*insert*)

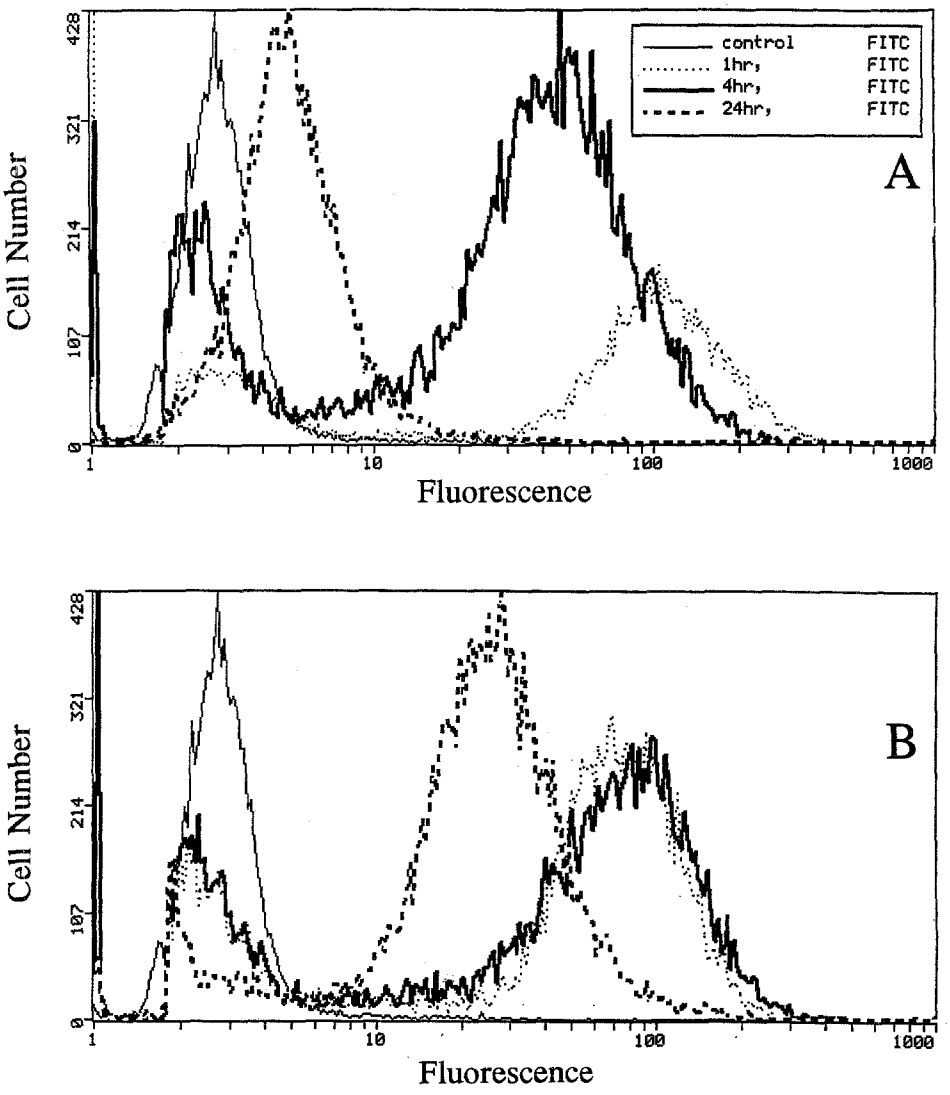

Fig. 4 A, B In vivo labeling of P388 cells with Ab injected i.p. Mice with established P388 tumours were injected i.p. with **A** 10 or **B** 100 μg of biotinylated anti-Thy 1.2 Ab in 0.5 ml HBSS. Controls received 0 μg Ab in 0.5 ml HBSS (–). At 1 (·····), 4 and (▬) and 24 (– – –) h, the animals were killed and the peritoneal cavity was lavaged with 5.0 ml HBSS. Lavge cells (10^6) were incubated with FITC-conjugated avidin (1 μg) for 30 min at 4 °C and examined for cell-associated fluorescence by flow cytometry

administration of mAb-SA-LUVs. Increased circulation lifetimes of SA-LUVs will increase the probability that the drug-loaded carrier will access extravascular target cells.

In these studies the procedure used to prepare SA-LUVs was modified such that the protein-liposome conjugates could be employed for delivery of a cytotoxic drug. The transmembrane pH-gradient loading procedure, developed for encapsulation of the anticancer drugs doxorubicin and vincristine, was used [6]. Briefly, 100-nm liposomes, composed of DSPC/chol/MPB-PE (54:45:1, mol ratio) were prepared in 300 m*M* citrate buffer (pH 4.0). The external pH was adjusted to 7.5 prior to streptavidin attachment to MPB-PE. SPDP-modified streptavidin (2–3 sulfhydryl groups per protein) was incubated with the liposomes as indicated in Materials and methods. As shown in previous reports, protein coupling promoted vesicle aggregation [24, 25]. Hence, the cross-linked streptavidin-coated liposomes were extruded through 100-nm pore size filters after the coupling reaction had been quenched by the addition of NEM. To ensure that the interior pH would remain at 4.0, the aggregated liposomes were passed down a Sepharose 4B-CL column equilibrated with 300 m*M* citrate buffer (pH 4.0) prior to extrusion. After the second sizing step, the resulting liposomes typically had 35–45 μg streptavidin/μmol lipid and exhibited a mean diameter of 130–170 nm as determined by QELS measurements.

The encapsulation and retention characteristics of SA-LUVs loaded with doxorubicin via the use of a transmembrane pH gradient (interior acidic) are summarised in Table 1. Greater than 98% doxorubicin encapsulation could be achieved within 2 min at an incubation temperature of 65 °C. The extent of drug uptake was not influenced by liposome-bound protein, and the doxorubicin-loading procedure did not effect the biotin-binding activity of SA-LUVs (results not shown). The resulting drug-loaded SA-LUVs exhibited drug retention characteristics similar to those of control liposomes, showing no significant drug release (over 4 h) in vitro (10% serum) or in vivo after i.v. injection.

An important characteristic of the doxorubicin-loaded liposomes, illustrated by the data in Fig. 2, concerns their increased circulation lifetime. Similar to protein-free liposomes [7], encapsulation of doxorubicin in SA-LUVs

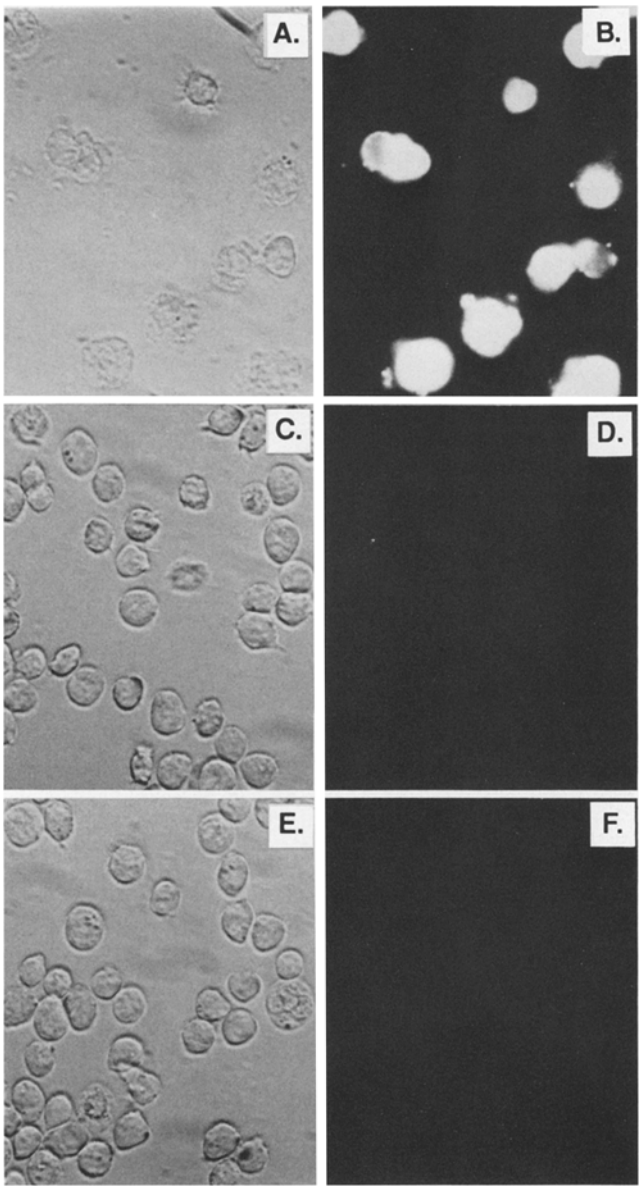

Fig. 5A–F Phase-contrast and fluorescent micrographs of targeted liposomes in vitro. Liposomes contained doxorubicin, which fluoresces under an FITC filter. **A** Phase-contrast view of P388 cells (10^7 cells/ml) preincubated with biotinylated anti-Thy 1.2 Ab (12.5 n*M*) and subsequently incubated with doxorubicin-loaded SA-LUVs (2 m*M* lipid) for 30 min in vitro at 4 °C. **B** Fluorescence image of the field illustrated in **A**. **C** Phase-contrast view of P388 cells treated as described above but without preincubation with Ab. **D** Fluorescence image of the field illustrated in **C**. **E** Phase-contrast view of P388 cells preincubated with Ab and subsequently incubated with doxorubicin-loaded control liposomes (no streptavidin). **F** Fluorescence image of the field illustrated in **E**

promotes their circulation lifetime. At 24 h, there was 3-fold more lipid in the circulation as compared with that obtained with empty SA-LUVs. This increase in circulating blood levels is associated with a significant decrease in liver accumulation for the doxorubicin-loaded liposomes (data not shown).

The targeting potential of doxorubicin-loaded SA-LUVs was assessed using P388 cells labeled with biotinylated

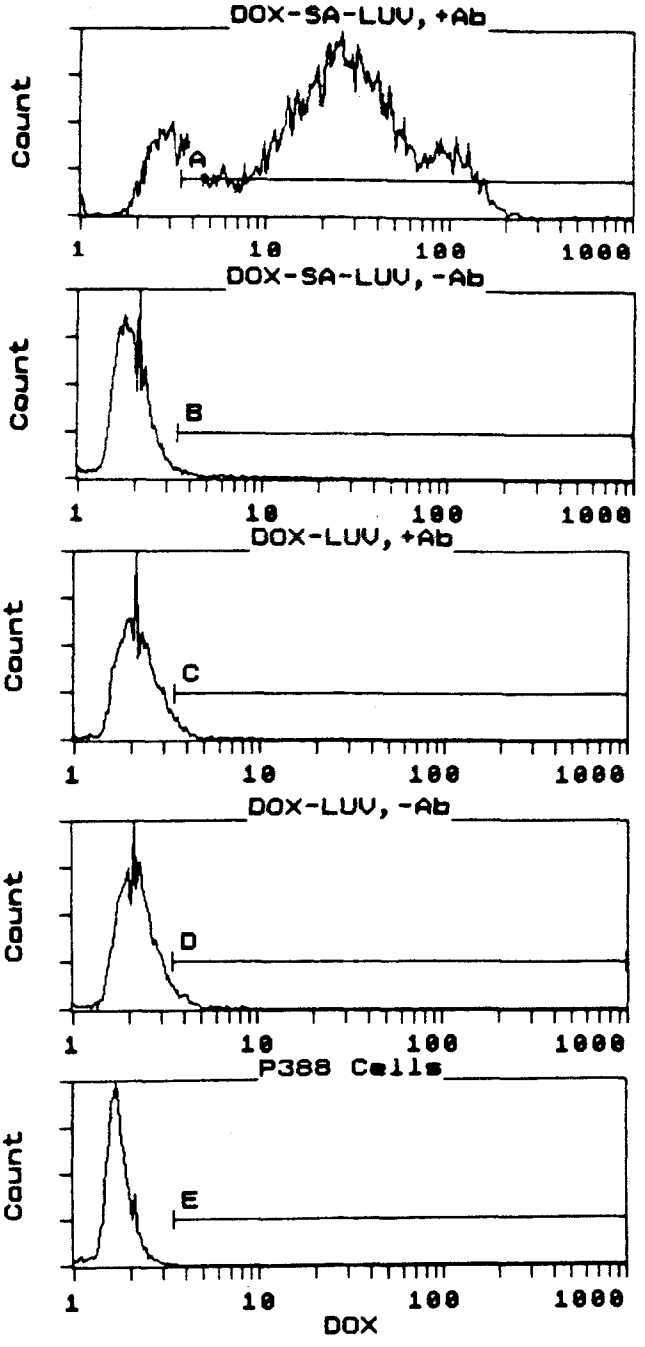

Fig. 6A–E Targeting of streptavidin liposomes via biotinylated anti-Thy 1.2 antibody to P388 cells in vitro. Doxorubicin-loaded SA-LUVs and LUVs were prepared as described in Materials and methods. P388 cells (10^7/ml) were incubated with biotinylated anti-Thy 1.2 Ab (12.5 n*M*) where indicated for 30 min at 4 °C. After two washes with PBS, doxorubicin-loaded SA-LUVs or LUVs (2 m*M* lipid) were added as indicated, incubated for 30 min at 4 °C and washed. Samples were subsequently examined for cell-associated fluorescence by flow cytometry

anti-Thy 1.2 antibody as a model target-cell population. The murine Thy 1.2 antigen is a highly expressed T-cell antigen with a molecular weight of approximately 25,000 Da [20]. The extent of biotinylated anti-Thy 1.2 antibody binding to P388 cells is shown in Fig. 3. These data demonstrate that approximately 60,000 antibodies bind per P388 cell. In addition, the data indicate that once bound, these antibodies are not internalised (Fig. 3, insert). The apparent binding constant for this antibody as estimated from the data in Fig. 2 is 5.5×10^9 M^{-1}.

To develop the use of P388 cells as a model target site in vivo, it is important to demonstrate that these cells can also be labeled with the anti-Thy 1.2 Ab in vivo. The ability to label P388 cells stably in vivo with biotinylated anti-Thy 1.2 antibody is illustrated by the flow-cytometric data shown in Fig. 4. Briefly, biotinylated anti-Thy 1.2 Ab was injected (i.p.) at a dose of 10 μg (Fig. 4A) or 100 μg (Fig. 4B) into mice with established P388 tumours (see Materials and methods). At 1, 4 and 24 h the peritoneal cavity was lavaged and antibody labeling of peritoneal cells was assessed. P388 cells within the peritoneal cavity were gated for on the basis of size and granularity characteristics of cultured P388 cells. The presence of cell-associated antibody was determined by the addition of fluorescein isothiocyanate (FITC)-conjugated avidin. The flow-cytometric data shown in Fig. 4 demonstrate that the percentage of antibody-labeled cells is constant for periods of at least 24 h in animals injected with 100 μg antibody. Fluorescent-microscopy data support this conclusion (results not shown). In vivo targeting studies using streptavidin liposomes were therefore initiated in animals injected with 100 μg biotinylated antibody.

Prior to the initiation of in vivo targeting studies, in vitro liposome-targeting experiments were completed using P388 cells that had been preincubated with an excess of biotinylated antibody. Doxorubicin-loaded liposome targeting to P388 cells was assessed qualitatively using flow cytometry and fluorescent microscopy, where the presence of cell-associated doxorubicin was detected by fluorescence. Figure 5 shows fluorescent micrographs of P388 cells labeled in vitro with doxorubicin-loaded SA-LUVs. Virtually all cells appeared fluorescent when they had been prelabeled with biotinylated anti-Thy 1.2 Ab (Fig. 5B). In the absence of antibody or surface-associated streptavidin (Figs. 5D and 5F, respectively) little, if any, fluorescence was observed. Although not clearly shown in the photomicrographs, labeling was restricted to the outer membrane and there was no indication of capping. Photomicrographs of cells labeled at 37 °C with doxorubicin-loaded liposomes were comparable with those obtained for cells labeled at 4 °C (results not shown).

Flow-cytometric analysis of labeled P388 cells confirmed these results. As shown in Fig. 6, specific cell labeling was observed only in situations where drug-loaded SA-LUVs were mixed with cells prelabeled with biotinylated anti-Thy 1.2 antibody (Fig. 6A). These data indicate that in vitro, approximately 90% of the P388 cells were labeled with doxorubicin-loaded liposomes. This result is consistent with data obtained for cells labeled with FITC-labeled streptavidin, where 95% of all prelabeled P388 cells appeared as positive (results not shown).

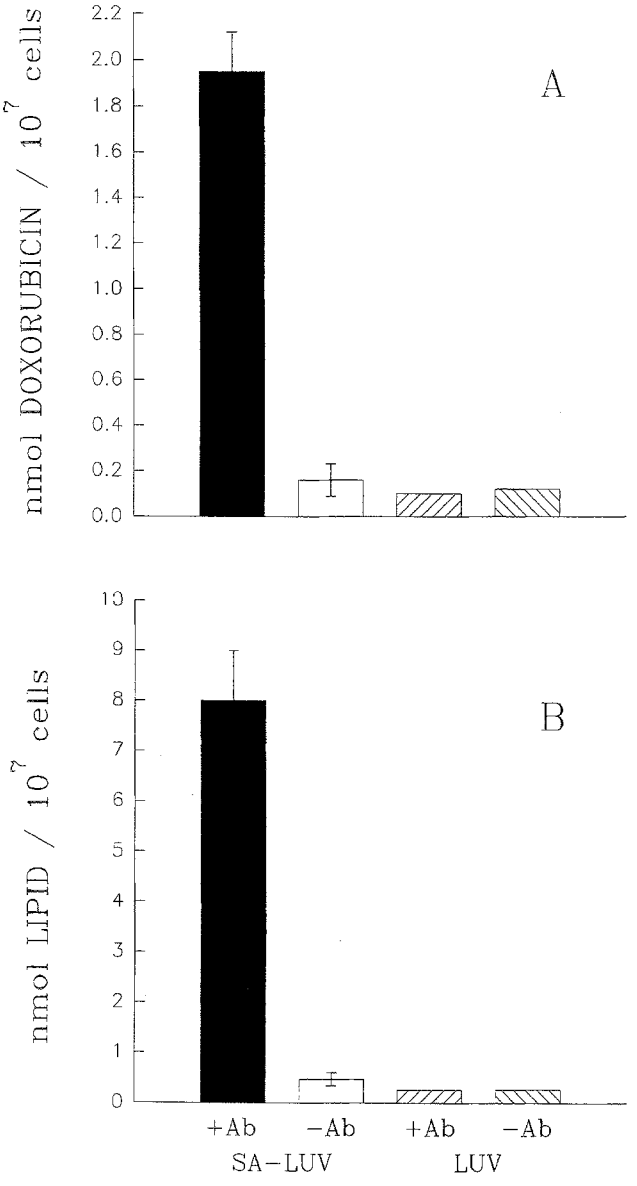

Fig. 7A,B Quantification of cell-associated doxorubicin and lipid after targeting of doxorubicin-loaded streptavidin liposomes to P388 cells in vitro. Doxorubicin-loaded SA-LUVs (40.0 μg streptavidin/μmol lipid) and LUVs were prepared as described in Materials and methods. P388 cells (10^7) were incubated with or without biotinylated anti-Thy 1.2 Ab (12.5 n*M*) as indicated for 30 min at 4 °C. After two washes with PBS, SA-LUVs (■, with Ab; □, without Ab) or LUVs (▨, with Ab; ▧, without Ab; 2 m*M* lipid) were added, incubated for 30 min at 4 °C and washed three times. Cell-associated amounts of **A** doxorubicin and **B** lipid were determined as detailed in Materials and methods

Quantitative determinations of cell-associated doxorubicin and cell-associated lipid are shown in Fig. 7. These data were obtained for P388 cells that had been incubated in vitro at 4 °C with or without biotinylated anti-Thy 1.2 antibody (10^7 cells suspended in 1 ml media at 12.5 n*M* Ab). The cells were washed to remove unbound antibody and resuspended in media with drug-loaded SA-LUVs or

Table 2 Concentrations of free and targeted liposomal doxorubicin producing 50% inhibition of tumour growth (IC_{50})

Sample	Antibody	IC_{50} (μM)[a]
Free drug	–	0.13
Streptavidin liposomal doxorubicin	–	10.1 ± 1.3
Streptavidin liposomal doxorubicin	+	3.8 ± 0.20

[a] IC_{50} values were obtained from P388 cells exposed for 24 h to doxorubicin-loaded streptavidin liposomes. Cytotoxicity assays were performed as described in Materials and methods

Table 3 In vivo targeting of streptavidin liposomes to P388 cells grown in the peritoneal cavity of BDF1 mice[a]

Route of administration	Sample	Antibody[b]	nmol lipid/10^7 cells (+/SEM)
i.p.[c]	LUV	–	1.60 ± 0.23
		+	1.51 ± 0.40
	SA-LUV	–	2.77 ± 0.24*
		+	5.70 ± 0.46**
i.v.[d]	LUV	–	1.89 ± 0.34
		+	1.80 ± 0.29
	SA-LUV	–	2.01 ± 0.30
		+	3.47 ± 0.42***

* Significantly different ($P < 0.05$) as compared with LUV controls (-Ab)

** Significantly different ($P < 0.01$) as compared with each control group

*** Significantly different ($P < 0.05$) as compared with each control group

[a] 1×10^6 P388 cells derived from a murine ascites tumour were injected i.p. (0.5 ml injection volume) 4 days prior to administration of 100 µg of the Thy 1.2 antibody

[b] 100 µg of Thy 1.2 antibody was injected i.p. in a volume of 500 µl

[c] DSPC/chol liposomal doxorubicin (LUV) and streptavidin DSPC/chol liposomal doxorubicin (SA-LUV) were injected i.p. 24 h after administration of antibody. At 1 h after liposome administration the animals were killed and the peritoneal cavity was lavaged as indicated in Materials and methods

[d] DSPC/chol liposomal doxorubicin (LUV) and streptavidin DSPC/chol liposomal doxorubicin (SA-LUV) were injected i.v. 24 h after administration of antibody. At 1 day after liposome administration the animals were killed and the peritoneal cavity was lavaged as indicated in Materials and methods

drug-loaded LUVs (without streptavidin). The final lipid concentration was 2 m*M*. Liposomal lipid and doxorubicin were assayed as described in Materials and methods. Using cells prelabeled with biotinylated anti-Thy 1.2 antibody, a 20-fold increase in cell-associated drug and a 30-fold increase in cell-associated lipid were obtained when cells incubated with drug-loaded SA-LUVs were compared with those incubated with protein-free liposomal doxorubicin. Non-specific binding of SA-LUVs to P388 cells that had not been incubated with antibody was typically less than 10% of the value obtained for targeted liposomes. Assuming 7×10^9 liposomes/nmol lipid (calculated on the basis of a mean diameter of 100 nm), it can be estimated under the conditions described herein that approximately 6,000 liposomes were bound per P388 cell. As indicated above (Fig. 3), about 60,000 antibodies bind per P388 cell when a saturating concentration of antibody is present.

The cytotoxic activity of doxorubicin loaded into targeted SA-LUVs was also assessed for cells exposed to drug for 24 h. The results, summarised in Table 2, indicate that the 50% growth-inhibitory concentration (IC_{50}) of free doxorubicin is almost 20-fold lower than that of the best liposomal drug. This was not unexpected, considering that the liposomes used were prepared from DSPC/chol, a composition known to retain drug for extended periods in vitro and in vivo (Table 1). Furthermore, where targeting was achieved it was mediated by binding to an antigen that is not readily internalised (Fig. 3). Given these constraints, it is surprising that drug-loaded SA-LUVs were 2- to 3-fold more active than doxorubicin entrapped in control liposomes (Table 2). These results suggest that the therapeutic activity of a liposomal drug can be improved through targeting even when the targeting ligand used is not internalised.

A summary of the in vivo targeting data obtained following i.p. and i.v. administration of doxorubicin-loaded SA-LUVs to P388 cells is provided in Table 3. For these studies 100 µg of biotinylated anti-Thy 1.2 Ab was injected i.p. into mice with established P388 tumours; 1 day later the mice were given an i.p. or i.v. injection of liposomes (3.3 µmol lipid/kg). Controls included protocols where streptavidin liposomes were injected into animals that had not received pre-injected Ab as well as injection of streptavidin-free liposomes into animals that had been pre-injected with the anti-Thy 1.2 antibody. Liposome binding to peritoneal cells was determined at 1 h after i.p. injection and 24 h after i.v. injection of SA-LUVs. The later time point was selected on the basis of previous studies that suggest that maximal liposome delivery to this site occurs at 24 h after i.v. administration [8]. As shown in Table 3, following i.p. injection of SA-LUVs there was a 3.8- and a 2.1-fold improvement in tumour cell-associated lipid delivery mediated by the presence of SA on the surface of the liposome or by pre-injection of biotinylated anti-Thy 1.2 Ab, respectively. The level of cell-associated lipid delivery was significantly lower than that observed in vitro (Fig. 5) and the specificity of cell delivery in vivo was at least 10-fold lower. This was primarily due to a 10-fold increase in non-specific binding to cells within the tumour.

Results obtained following i.v. injection of doxorubicin-loaded SA-LUVs (see Table 3) suggest that SA-mediated binding to biotin-labeled P388 cells can result in a 1.7- to 1.9-fold increase in cell association. However, the specificity is significantly lower than expected on the basis of in vitro studies. Flow-cytometric analysis of peritoneal cells selected on the basis of characteristics of culture P388 cells (see Materials and methods) suggested that only a small percentage (<20%) of the cells were specifically labeled with doxorubicin-loaded liposomes as a result of pre-injection of the biotinylated anti-Thy 1.2 Ab (Fig. 8). This finding is in contrast to the results shown in Fig. 6, where greater than 90% of the cells were labeled with

liposomes in vitro. It should also be noted that the percentage of injected SA-LUVs reaching the peritoneal cavity at 1 day after i.v. administration (3%) was not increased significantly under conditions where the P388 cells were well labeled with the biotinated Thy 1.2 antibody (results not shown). More specifically, the presence of a defined target population in the peritoneal cavity did not promote accumulation of liposomes in this extravascular site. The antibody did, however, facilitate a redistribution of liposomes within that site, thus increasing the level of cell-associated liposomes.

Discussion

Previous reports from this laboratory have described the preparation and characterisation of a versatile liposome-targeting approach that relies on the high-affinity binding of biotin to streptavidin [23–25]. The studies described herein demonstrate that this targeting approach can be used to target doxorubicin-loaded liposomes to murine tumour cells both in vitro and in vivo. Furthermore, we show using in vitro cytotoxicity assays that doxorubicin entrapped in these targeted liposomes is more cytotoxic than doxorubicin encapsulated in non-targeted liposomes. The results lead to several conclusions regarding the design of liposomes for in vivo targeting, and these are discussed below.

The first point concerns the use of two-step targeting approaches for development of liposomal anticancer agents given by i.v. administration. Our work is based on the premise that to achieve targeting to cells within an extravascular site, the liposomes must first be capable of efficiently moving from the blood compartment to an interstitial space within the target site. Studies using murine models suggest that the circulation lifetimes of the carrier must be maximized to achieve efficient delivery of entrapped contents to tumours [12, 28]. An advantage of the SA-LUVs used in the present study is that they exhibit circulation lifetimes comparable with those of liposomes that do not have associated protein [24]. In the case of antibody-coated liposomes, rapid clearance from the plasma compartment has been observed following i.v. administration [11]. Similar results are presented herein for mAb-SA-LUVs prepared using the avidin-biotin bridge technique (see Fig. 1). Clearance of these immunoliposomes may be partly attributed to liposome aggregation; however, the data presented in Fig. 1 were obtained using liposomes exhibiting identical mean diameters (approximately 170 nm). The presence of surface-associated antibody clearly decreased the circulation lifetime of the liposomes.

It is important to demonstrate in vitro that the two-step targeting procedure can be as efficient as direct approaches involving antibody-coated liposomes. Previous reports indicate that mAb-coated liposomes can result in a 2- to 28-fold increase in target-cell association over control liposomes [1, 9, 16, 21, 26, 29]. The data presented herein demonstrate that the two-step targeting procedure, based on

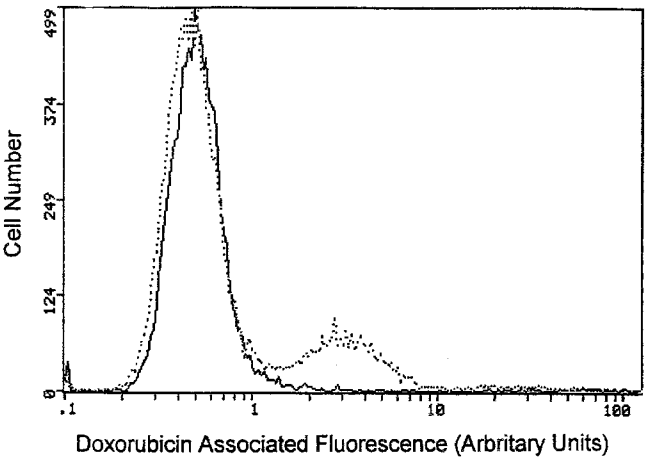

Fig. 8 Flow-cytometric analysis of P388 cells labeled in vivo following i.v. administration of streptavidin-labeled liposomal doxorubicin into mice that had been preinjected with saline (*solid line*) or biotinylated anti-Thy 1.2 antibody (*dashed line*) as described in Table 3 and Materials and methods

binding SA-LUVs to P388 cells prelabeled with biotinylated anti-Thy 1.2 antibody, results in a 30-fold increase in liposomal lipid association over controls. A limitation of the two-step procedure, however, concerns the observation that optimal targeting requires the use of a target antigen that is not internalised. We chose the Thy 1.2 antigen as a target ligand for this reason. Thy 1.2 is a glycolipid-anchored, low-molecular-weight (25 kDa) protein that is highly expressed on P388 cells. The biotinylated antibody used for these studies exhibits a relatively high binding affinity (5.5×10^9 M^{-1}) and, once bound, the rate of antibody loss from the cell surface is low (Fig. 2, insert). This retention of surface-associated antibody was also maintained in vivo (Fig. 4).

In vivo targeting to prelabeled target cells was also demonstrated for doxorubicin-loaded SA-LUVs injected i.p. and i.v. Liposomes injected i.p. exhibited a 2.5-fold increase in cell-associated lipid and drug when the cells were prelabeled with Ab (Table 3). A significant portion of the non-specific cell-associated lipid and drug can probably be attributed to liposome uptake by macrophages or other phagocytic cells in the peritoneum of P388 tumour-bearing animals. This conclusion is supported by flow-cytometric results (Fig. 8) where P388 cells within the peritoneal cavity were tentatively identified on the basis of the size and granularity characteristics of cultured P388 cells. There was negligible non-specific cell-associated fluorescence (due to doxorubicin) in this defined cell population, suggesting that cell-associated drug was localized primarily in a cell population other than the P388 cells. In contrast, however, approximately 20% of the gated cell population was shown to be positive for liposome-associated doxorubicin when isolated from animals pre-injected with Ab. This percentage is significantly lower than that observed in vitro, where a 20- to 30-fold Ab-mediated increase in cell-associated drug and lipid, respectively, was observed (see Fig. 6).

The in vivo results suggest that additional problems must be addressed so as to optimize liposomal targeting. In particular, liposome characteristics optimized on the basis of in vitro targeting studies may have to be reassessed in vivo. The results shown in Table 3 suggest that not only the specificity of cell binding is decreased, but the efficiency of binding is significantly reduced. This was observed even under conditions where there was a vast excess of SA-LUVs present (i.p. injected SA-LUVs) and when the binding reaction was mediated by biotin binding to streptavidin (Ka, 10^{15} M^{-1}). It was also surprising that the presence of a well-labeled target cell population residing in an extravascular site did not promote accumulation in this region following i.v. administration. This was anticipated on the basis of extravasation models proposing that an equilibrium is established between liposomes in the blood compartment and liposomes in the interstitial space of a defined tissue or region. The results presented herein suggest that further improvements in targeting will be achieved by optimizing the binding avidity of target liposomes for a target ligand and through procedures that promote extravasation of i.v. injected liposomal carriers.

Acknowledgements This research was supported by the B.C. Health Research Foundation and the Medical Research Council of Canada. M.B.B. is a B.C. Health Research Foundation Scholar. We thank L. Wood and C. Bay for assistance in the preparation of this manuscript.

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