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A Model Approach for Assessing Liposome Targeting *in Vivo*

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A procedure intended to facilitate characterization and optimization of liposomes designed for *in vivo* targeting to sites outside the blood compartment is described. The approach is based on a model consisting of administering streptavidin liposomes intravenously to mice previously injected intraperitoneally or intratumorally with biotinylated multilamellar vesicles (MLVs). *In vivo* targeting, therefore, is measured through the evaluation of streptavidin liposome accumulation and distribution within the site of MLV injection. *In vitro* studies suggested that optimal binding would be achieved when streptavidin liposomes, prepared with 2 mol% polyethylene glycol-modified phospholipids (PEG-SA-LUV), were incubated with multilamellar vesicles incorporating biotinoylaminohexanoyl DSPE (BAH-MLV). *In vivo* targeting studies were focused in three areas. The least stringent test determined PEG-SA-LUV binding to biotinylated MLVs in the peritoneal cavity after ip administration and resulted in a 17-fold increase in binding of PEG-SA-LUVs to MLVs within the peritoneal cavity 24 h after injection. Alternatively, a 5-fold increase in binding to MLVs was achieved in animals when the PEG-SA-LUVs were administered intravenously. The third approach consisted of iv administration of PEG-SA-LUVs into mice bearing subcutaneous Lewis lung tumors that had been injected with either BAH-MLVs or, in a contralateral tumor, control MLVs. Under these conditions a 2-fold increase in tumor accumulation was achieved in tumors injected with the biotinylated MLVs. The results presented indicate that approaches designed to facilitate targeting of liposomal drugs to extravascular sites will result in little or no change in the capacity of these liposomes to accumulate passively. © 1995 Academic Press, Inc.

Target-specific delivery of liposomes *in vivo* has been the objective of many studies aimed at improving the therapeutic index of liposomal drugs. Ligand-mediated targeting of liposomes to cells has been achieved using monoclonal antibodies (1–3), avidin and streptavidin (4–6), transferrin (7, 8), as well as other ligands that bind to selected cell surface markers. Several of these studies have demonstrated *in vitro* that a high-affinity interaction between the targeting ligand and the surface marker must be maintained for optimal targeting. The apparent binding avidity of liposomes is controlled by several factors including the type of chemical modification required to couple the targeting ligand to the liposome surface (9) as well as liposome surface characteristics. With regard to the latter, nonspecific binding to cell surfaces must be minimal while target ligand/receptor interaction should be maximized. Incorporation of PEG-modified lipids into liposomes can, for example, inhibit liposome–cell binding. However, the presence of this molecule is also known to reduce binding of liposome-associated targeting ligands to a target surface. Reductions in binding of liposomes to the target cell population *in vitro,* however, may be compensated for through addition of more targeting ligands to the liposomal surface (1, 10, 11).

In addition to the characteristics of the targeted liposome, the nature of the cell-associated marker will be important to the success of any targeting approach. Targeting has been achieved through both internalized and noninternalized surface markers (6, 7). In general, however, these target molecules must be expressed at reasonably high levels (>10,000 copies per cell) in order to achieve optimal binding. If targeting is mediated through an internalized target receptor, then vesicle size must be such that normal internalization processes can occur. Transferrin bound to the transferrin receptor, for example, is internalized by clatherin-coated endocytic vesicles that exhibit a mean diameter

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of less than 100 nm (12). Liposomes targeted to this receptor should not exceed this size.

In addition to maintaining cell surface specificity, liposomes used for *in vivo* targeting must be able to access the target cell population. Successful *in vivo* targeting of liposomes has been achieved using monoclonal antibodies specific for lung endothelial cells (13) as well as for tumour cells that seed within the lung (14). A therapeutic advantage has been demonstrated for the latter when employing doxorubicin-loaded antibody-coated liposomes (14). If the target cell population resides outside the vascular compartment, then the *in vivo* targeted liposomal formulations must exhibit characteristics that promote extravasation to the disease site. Although the mechanisms responsible for this extravasation process are not well understood, it is established that liposome size and circulation lifetime are critical determinants (15–17). It has been documented, for example, that liposomes without surface-associated targeting information can access extravascular sites in diseased tissues such as tumors (15, 16, 18–20). Optimal delivery of liposomes and associated contents requires use of liposomes that: (1) exhibit mean diameters of less than 200 nm; (2) are retained in the plasma compartment for extended periods; and (3) are capable of retaining associated contents following iv administration.

The many factors described above make it difficult to optimise liposomes for *in vivo* targeting. We have therefore developed a model approach where the target consists of biotin-labeled multilamellar liposomes (MLVs). The primary advantages achieved through use of biotin-labeled MLVs as a target population include elimination of variables related to antigen density, antibody/antigen turnover, and cell number. The studies presented here, therefore, focus specifically on factors that define access and binding of liposomes with a target. In this model system the targeted liposomes have surface-associated streptavidin to facilitate binding. It is demonstrated that biotin-labeled MLVs are stable *in vivo,* are retained well within the injection sites, and can effectively bind PEG-SA-LUVs after intravenous administration.

MATERIALS AND METHODS

Materials

Distearoylphophatidylcholine (DSPC) was obtained from Avanti Polar Lipids and *N*-succinimidyl 3-(2-pyridyldithio)-propionic acid (SPDP) was from Molecular Probes. Doxorubicin was obtained from Adria Laboratories of Canada (Mississauga, Ontario). Normal mouse serum was purchased from Cedarlane Laboratories (Hornby, Ontario). Cholesterol, dithiothreitol (DTT), β-mercaptoethanol, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), *N*-ethylmaleimide (NEM), streptavidin, Sephadex G-50, D-biotin, Sepharose CL-4B, and all salts were obtained from Sigma. [^{3}H]Cholesterol hexadecyl ether and [^{14}C]cholesterol hexadecyl ether were obtained from NEN and [^{14}C]biotin was from Amersham. *N*-(4-(*p*-maleimidophenyl) butyryl) dipalmitoylphosphatidylethanolamine (MPB-DPPE), *N*-biotinoyldistearolphosphatidylethanolamine (B-DSPE), *N*-biotinoylaminohexanoyldistearoylphosphatidylethanolamine (BAH-DSPE), and polyethylene glycol-modified phosphatidylethanolamine (PEG-DSPE) were 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.* (1985) (21). Briefly, lipid films (52 mol% DSPC, 45 mol% cholesterol, 2 mol% PEG_{2000}- DSPE, 1 mol% MPB-DPPE) 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 mM citrate (pH 4.0) by vortex mixing such that a final lipid concentration of 50 mM was achieved. LUVs were then prepared by extrusion (five times) at 65°C through three 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) preequilibrated with 150 mM NaCl, 25 mM Hepes, pH 7.5 (HBS). Multilamellar vesicles (MLVs) were prepared by hydrating the lipid film (54 mol% DSPC, 45 mol% cholesterol, 1 mol% biotinoylaminohexanoyl DSPE) at 65°C in HBS by vortex mixing such that a final lipid concentration of 150 mM was achieved. MLVs were washed twice in HBS (3000 rpm × 10 min) to remove any LUV or small unilamellar vesicles (SUVs) which formed during hydration. Typically, liposomes were prepared such that the nonexchangeable, nonmetabolizable lipid [^{3}H]cholesteryl hexadecyl ether (for LUVs) or [^{14}C]cholesteryl hexadecyl ether (for MLVs) was incorporated as a liposome label for quantification in both *in vitro* and *in vivo* studies.

SPDP Modification of Streptavidin

Streptavidin (5 mg/ml in HBS) was modified with the amine reactive agent SPDP according to published pro-

cedures (22). Briefly, SPDP (25 mM 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 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) 10 min after addition of DTT according to published procedures (23).

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.* (1990) (22). Briefly, SPDP-streptavidin was incubated with liposomes at a ratio of 75 μg protein/μmol lipid (10 mM final lipid concentration). The coupling reaction was stopped after 5 min by the addition of dithiothreitol (DTT) (10 times molar excess over MPB-PE) followed 2 min later by the addition of *N*-ethylmaleimide (NEM) (10 times molar excess over DTT + SPDP), and unassociated protein was removed by gel filtration on Sepharose CL-4B equilibrated with HBS. The extent of streptavidin coupling to liposomes was determined using a functional assay which measured binding of [^{14}C]biotin to streptavidin. Briefly, streptavidin-coated liposomes (0.50 μmol lipid in 0.5 ml) were incubated with [^{14}C]biotin (7.31 nmol added, 46.9 nmol/mCi) for 10 min and 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-coated liposomes (5–10 mM 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 streptavidin-coated liposomes by column chromatography using Sephadex G-50 preequilibrated with HBS.

Separation of MLVs and LUVs

MLVs (20 μmol lipid) and LUVs (0.5 μmol lipid) were incubated together at RT for 30 min (1.0 ml final volume). Samples were spun at 3000 rpm (1600*g*) for 10 min and the supernatant was collected. After three washes with HBS (1600*g* × 10 min) the pellet was taken up in 1.0 ml HBS. Both supernatant and pellet were assayed for lipid as described later.

Animals

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 ip dose (500 μl volume) or an iv dose (200 μl volume) via the lateral tail vein. Blood was collected via cardiac puncture and placed in EDTA-treated microtainers (Becton-Dickinson, Canada). Plasma was prepared by centrifuging (200*g*) blood samples for 10 min in a clinical centrifuge. Total plasma volume per animal was taken to be 4.55% of mean body weight. Peritoneal cavities were lavaged with 5 ml of indicator-free and Ca^{2+}-, Mg^{2+}-free Hanks' buffed saline solution (HBSS) which was injected ip. 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. BDF1 mice bearing P388 tumors were prepared by inoculating mice ip with 1 × 10^6 P388 cells. Tumors progressed for 4 days prior to injection of the specified liposomal preparation. Four days after cell administration there was no measurable ascites fluid. Lavage fluid which contained red cell contamination was discarded. BDF1 mice bearing Lewis lung tumors were prepared by inoculating mice subcutaneously with 3 × 10^5 Lewis lung cells (obtained from the NCI tumor repository). Tumors progressed for 10–13 days prior to injection of liposomes.

Quantitation of Lipid

Liposomal lipid was measured by incorporation of tracer quantities of the nonexchangeable, nonmetabolizable radiolabeled lipid marker [^{3}H]cholesterol hexadecyl ether (24). 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.

RESULTS

The studies described here use biotin-labeled MLVs as a model target that can be specifically labeled with streptavidin liposomes (PEG-SA-LUVs). PEG-SA-LUV binding to biotin-MLVs was assayed after separating MLVs from LUVs by low-speed centrifugation. Data obtained using this procedure have been summarized in

TABLE 1

Separation of LUVs and MLVs

Sample	Supernatant (μmol lipid)		Pellet (μmol lipid)	
	MLVs (^{14}C)	PEG-SA-LUV (^{3}H)	MLVs (^{14}C)	PEG-SA-LUV (^{3}H)
PEG-SA-LUVs (0.5 μmol lipid)	0.000	0.491	0.000	0.000
Biotin-MLVs (20 μmol lipid)	0.009	0.000	19.713	0.000
LUVs (0.5 μmol) + biotin-MLVs (20 μmol)	0.000	0.493	19.801	0.000
PEG-SA-LUVs (0.5 μmol) + MLVs (20 μmol)	0.000	0.489	19.752	0.000
PEG-SA-LUV (0.5 μmol) + biotin-MLVs (20 μmol)	0.000	0.009	19.846	0.490

Note. All incubations were done at RT for 30 min in a 1.0-ml volume, at which time samples were spun at 3000 rpm (1600*g*) for 10 min. MLV and LUV lipid present in the pellet and supernatant were determined as described under Materials and Methods.

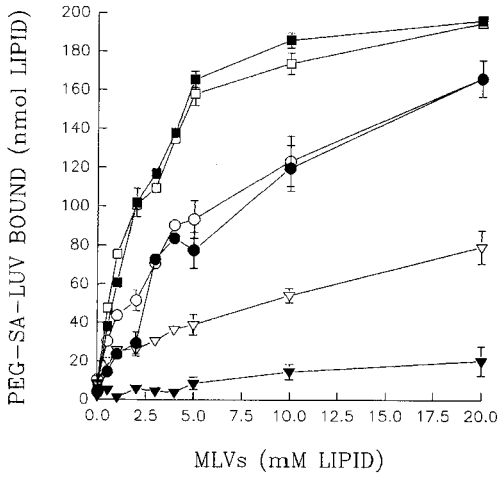

FIG. 1. Influence of serum on the binding of PEG-SA-LUVs to MLVs incorporating different biotin-labeled phospholipids. PEG-SA-LUVs (0.2 mM) were incubated at RT for 30 min with MLVs incorporating B-DOPE (▼), B-DSPE (●), or BAH-DSPE (■) in the presence (solid symbols) or absence (empty symbols) of 10% normal mouse serum. Quantification of bound PEG-SA-LUV was determined as under Materials and Methods. Values shown represent the mean from at least four experiments ± SD.

Table 1. Briefly, ^{3}H-labeled PEG-SA-LUVs, spun at 1600*g* for 10 min, do not pellet. In contrast, ^{14}C-labeled biotin-MLVs pellet efficiently with greater than 98% of the lipid recovered in the pellet after three washes. When protein-free [^{3}H]LUVs (with 2 mol% PEG-modified DSPE) were mixed with ^{14}C-labeled biotin-MLVs or, conversely, when [^{3}H]PEG-SA-LUVs were mixed with [^{14}C]MLVs (no biotin) there was complete separation of the MLVs (98% recovered in the pellet) and LUVs (98% recovered in the supernatant). Specificity of binding is demonstrated when PEG-SA-LUVs are mixed with biotin-MLVs. This results in greater than 98% of LUVs to copellet with the MLVs.

The centrifugation assay described above was used in initial studies designed to select a biotin-labeled lipid with optimal binding characteristics. Three different biotinylated lipids were used including: (1) an unsaturated biotin-labeled phospholipid derived from DOPE (B-DOPE); (2) a saturated biotin-labeled phospholipid derived from DSPE (B-DSPE); and (3) a saturated biotin-labeled phospholipid where the biotin was coupled to DSPE via a six-carbon hexanoyl spacer arm (BAH-DSPE). The biotinylated lipid was always incorporated at the level of 1 mol% in the MLVs and PEG-SA-LUV binding was determined as a function of MLV concentration (Fig. 1). In addition, binding of PEG-SA-LUVs to biotin-MLVs was determined in the presence and absence of 10% serum. Three important conclusions can be made on the basis of the data shown in Fig. 1. First, PEG-SA-LUV binding to MLVs containing B-DOPE is significantly inhibited by the presence of serum. Second, in the absence of serum the binding of PEG-SA-LUVs to MLVs containing B-DSPE is significantly greater than results obtained using MLVs with B-DOPE and binding is no longer inhibited by serum. Acyl chain composition, therefore, is clearly an important feature defining the avidity of binding in this model. Third, binding of PEG-SA-LUVs to biotin-MLVs was most efficient when the MLVs incorporated biotinoylaminohexanoyl DSPE (BAH-DSPE). The concentration of MLVs required to obtain 50% binding of the PEG-SA-LUVs was decreased almost fourfold when the biotin was attached via the six-carbon spacer arm in BAH-DSPE. The data in Fig. 1 suggest that the most efficient binding of PEG-SA-LUV occurs when using MLVs with BAH-DSPE; therefore, studies described from this point forward focus on targeting to these MLVs, hereafter referred to as BAH-MLVs.

In an effort to establish the binding capacity of BAH-MLVs, a study assessing binding as a function of PEG-SA-LUV concentration was completed. The results, shown in Fig. 2, were obtained in the presence of serum using BAH-MLV concentration of 20 μmol/ml. Under these conditions a maximum of 1.3 μmol of PEG-SA-LUV lipid can bind to 20 μmol of BAH-MLV lipid in a 1-ml assay volume. It is important to note that at concentrations up to 800 μM PEG-SA-LUV essentially 100% of the added LUVs bound to the BAH-MLVs. Binding specificity between PEG-SA-LUVs and BAH-

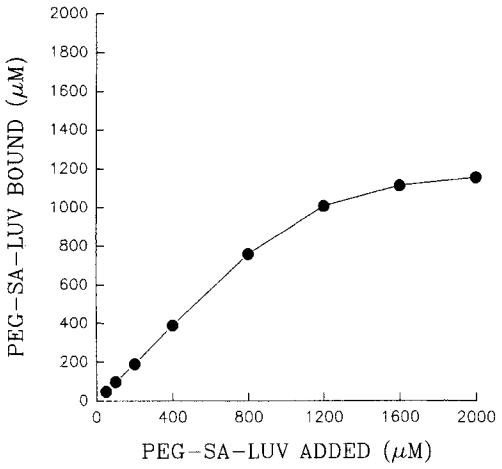

FIG. 2. Binding of PEG-SA-LUVs to BAH-MLVs. BAH-MLVs (20 mM) were incubated at RT for 30 min with various concentrations of PEG-SA-LUVs. Quantification of bound PEG-SA-LUV was determined as under Materials and Methods.

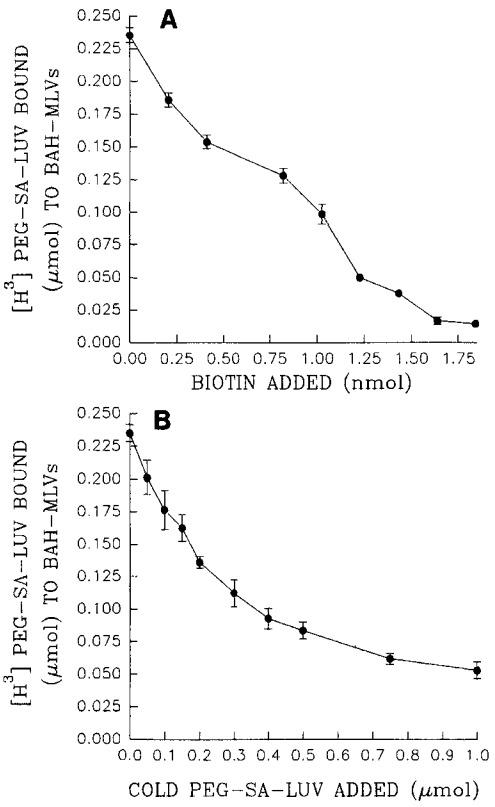

FIG. 3. Competitive inhibition of PEG-SA-LUV binding to BAH-MLVs with free biotin or PEG-SA-LUVs. Various concentrations of free biotin or cold PEG-SA-LUV were incubated with BAH-MLVs (20 mM) and [H³]PEG-SA-LUV (0.5 mM) at RT for 30 min. Quantification of bound PEG-SA-LUV was determined as under Materials and Methods. Values shown represent the mean from at least four experiments ± SD.

MLVs was measured through competitive inhibition studies summarized in Fig. 3. The results demonstrate that addition of free biotin (Fig. 3A) completely inhibits binding at concentrations of 1.65 mM. At this concentration of biotin there are approximately 2.5 free biotins per streptavidin molecule bound to the LUVs, a result that is consistent with previous studies demonstrating that liposome bound streptavidin is capable of binding 2 to 3 mol biotins per mole of protein (5). Further, the results in Fig. 3B demonstrate that the binding reaction between radiolabeled PEG-SA-LUVs and BAH-MLVs is readily inhibited by addition of excess levels of nonradiolabeled PEG-SA-LUVs. A fivefold decrease in binding is observed when the radiolabeled liposomes are diluted fivefold with "cold" PEG-SA-LUVs.

The results presented to this point demonstrate that BAH-MLVs can serve as an appropriate target for PEG-SA-LUVs. The reaction is specific (biotin and/or cold PEG-SA-LUV addition blocks binding), there is negligible background binding (little measurable binding to biotin-free MLVs), and addition of serum proteins does not effect binding. The following studies address whether this model targeting system can be used *in vivo*. First, it is important to demonstrate that PEG-SA-LUVs recovered from the blood following iv administration can still bind BAH-MLVs. This study, illustrated in Fig. 4, used PEG-SA-LUVs isolated from the circulation of mice following iv administration at a dose of 100 mg lipid/kg (3.29 μmol/mouse). Plasma, with liposomal lipid, was isolated at 1 and 24 h after injection. Subsequently, a sufficient volume of plasma containing 200 μmol of PEG-SA-LUVs was added to various concentrations of BAH-MLVs. Following an incubation time of 30 min at room temperature the MLVs were separated from nonassociated PEG-SA-LUVs by centrifugation as described above. The binding of PEG-SA-LUVs isolated 1 h after administration was identical to control liposomes that had not been injected. There was a slight, yet significant, decrease in PEG-SA-LUV binding when the liposomes were recovered 24 h after administration. As indicated in Fig. 4, 50% binding of PEG-SA-LUVs isolated from plasma 24 h after administration required twice as much MLV lipid as controls. It is important to note that binding between BAH-MLVs and PEG-SA-LUVs isolated from animals maintained on an avidin-enriched, biotin-depleted diet for 5 days was identical to the data shown in Fig. 4 (data not shown). It can be suggested, therefore, that endogenous biotin levels will not affect binding.

A final consideration as to whether the model approach described here will be of use concerns whether the target BAH-MLVs maintain binding activity after *in vivo* administration and whether this target is retained well within the site of injection after administration. For these studies BAH-MLVs or control MLVs were injected ip and the level of liposomal MLV lipid

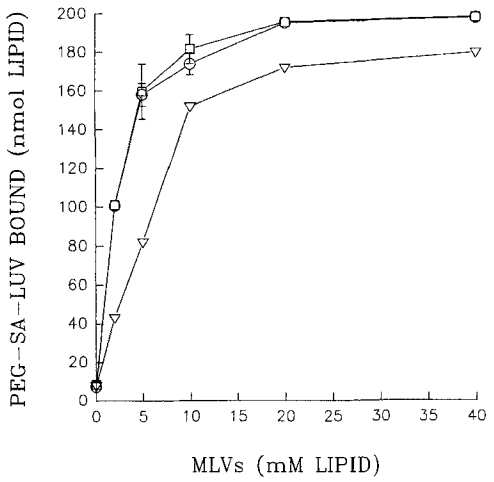

FIG. 4. Influence of *in vivo* incubation of PEG-SA-LUV in the mouse blood compartment on binding to BAH-MLVs *in vitro*. PEG-SA-LUV was injected via lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg/kg lipid). The mice were euthanized at 1 and 24 h after injection and liposomal lipid in the plasma was recovered and measured as under Materials and Methods. Recovered PEG-SA-LUVs (0.5 mM) (□, 0 h; ○, 1 h; ▽, 24 h) were incubated with BAH-MLVs (20 mM) at RT for 30 min. Quantification of bound PEG-SA-LUV was determined as under Materials and Methods. Values shown represent the mean from at least two animals ± SE of the mean.

in the peritoneal cavity was determined 1 day later. Subsequently, the isolated MLVs were used to evaluate PEG-SA-LUV binding. The results, summarized in Table 2, indicate that for both control MLVs and BAH-MLVs greater than 85% of the injected (ip) dose of lipid is retained within the peritoneal cavity 24 h after administration. Further, the ability of PEG-SA-LUVs to bind isolated BAH-MLVs is identical to that observed for BAH-MLVs that were not injected.

Several approaches were taken to demonstrate how this model approach can be used to evaluate liposome targeting *in vivo*. In general, PEG-SA-LUVs (or control, streptavidin-free, LUVs) were administered either iv or ip at a dose of 3.29 μmol per mouse (100 mg lipid/kg) 1 h after an ip injection of BAH-MLVs (or control, biotin-free, MLVs) given at a lipid dose of 20 μmol per mouse. In order to evaluate *in vivo* targeting under conditions where vascular permeability was enhanced these studies were done in animals with and without established P388 ascitic tumors. The presence of an ascitic tumor has been shown to increase access of circulating macromolecules (25, 26) and liposomes (20) to the peritoneal cavity. The assay for targeting was based on measuring: (1) the amount of LUVs recovered in the peritoneal cavity 24 h after administration and (2) the amount of recovered PEG-SA-LUVs that could be pelleted at 1600*g* after a 10-min centrifugation. Under these conditions the pelleted material consists of MLVs as well as peritoneal cells. Therefore, flow cytometry was utilized to distinguish whether the PEG-SA-LUV binding was to the target MLVs or to cells. Finally, it should be noted that these studies used PEG-SA-LUVs or control-LUVs that were loaded with the anticancer drug doxorubicin. Entrapped doxorubicin was used for the following reasons: (1) doxorubicin is a convenient fluorescent molecule that can readily be evaluated by flow cytometry and a quantitative fluorescence drug assay; (2) the presence of doxorubicin in the LUVs increases liposome circulation longevity (27); and (3) our primary research objective is to develop procedures that result in more specific delivery of anticancer drugs to sites of tumor growth.

TABLE 2

Retention of Vesicles Injected in the Peritoneal Cavity

Liposomes	% Recovery in peritoneal cavity 24 h after administration (− tumor)	Concentration of BAH-MLV to achieve 50% of maximum PEG-SA-LUV binding
MLV	88 ± 3%	N.A.
BAH-MLV	85 ± 3%	2.5 mM
LUV	2.2 ± 2.0%	N.A.

Note. BAH-MLVs, MLVs, or LUVs (20 μmol lipid) were injected ip in mice. Vesicles were recovered by lavage 24 h later and assayed for their ability to bind PEG-SA-LUVs as under Materials and Methods. Values shown represent the mean from at least four animals ± SD.

Results from these initial *in vivo* targeting studies, summarized in Table 3, illustrate several points. First, following ip injection of LUVs in mice preinjected ip with BAH-MLVs (see Table 3, group A) one observes significant retention of injected LUV lipid only when LUVs are coated with SA. In the absence of tumor, almost 40% of the injected PEG-SA-LUV dose was recovered 24 h following administration, whereas less then 1% of the control (no streptavidin) LUV dose was recovered. Retention was dependent on use of MLVs with surface associated biotin. Furthermore, flow cytometric analysis, where differences between liposomes and cells were based on forward light scattering characteristics, suggested that the pellet associated PEG-SA-LUVs were bound primarily to MLVs and not peritoneal cells (results not shown). It should be noted that macrophages within the peritoneal cavity were exposed to 20 μmol MLV lipid for 24 h prior to injection of the 3.29 μmol LUVs, therefore most likely inhibiting uptake of the LUVs.

In the presence of an established ascitic tumor there is an increase in the retention of ip administered LUVs, even in the absence of appropriate targeting ligands (see Table 3, group B). This result is consistent with studies that suggest that established peritoneal tumors can block lymphatic drainage, the primary mechanism responsible for elimination of small (<200 nm) liposomes from the peritoneal cavity (28, 29). Retention of LUVs injected ip, for example, increased from less than

TABLE 3

In Vivo Targeting of SA-LUVs to BAH-MLVs

Group	LUV type	Route of administration	MLV type (injected ip)	Ascitic tumor	Total LUV lipid recovered in pc (nmol)	Total LUV lipid pelleted (nmol)	Fold improvement over control
A	SA-LUVs	ip	BAH-MLVs	−	1138 ± 170	502 ± 86	17.9-fold
	SA-LUVs	ip	MLVs	−	32 ± 20	28 ± 18	—
	LUVs	ip	BAH-MLVs	−	0 ± 0	0 ± 0	—
B	SA-LUVs	ip	BAH-MLVs	+	1381 ± 100	1249 ± 207	8.4-fold
	SA-LUVs	ip	MLVs	+	499 ± 66	160 ± 22	—
	LUVs	ip	BAH-MLVs	+	540 ± 25	146 ± 39	—
C	SA-LUVs	iv	BAH-MLVs	−	27.3 ± 0.8	15.7 ± 1.0	5.8-fold
	SA-LUVs	iv	MLVs	−	26.8 ± 1.4	3.2 ± 0.4	—
	LUVs	iv	BAH-MLVs	−	26.0 ± 0.1	2.2 ± 0.8	—
D	SA-LUVs	iv	BAH-MLVs	+	121.1 ± 28.1	82.8 ± 14.9	6.2-fold
	SA-LUVs	iv	MLV	+	111.5 ± 13.7	11.8 ± 2.0	—
	LUVs	iv	BAH-MLVs	+	157.7 ± 6.7	17.8 ± 11.1	—

Note. Three days after ip injection of 10^6 P388 cells (where indicated), MLVs were injected ip at a dose of 20 μmol lipid/mouse. SA-LUVs were injected ip or via lateral tail vein at a dose of 3.29 μmol lipid/mouse (100 mg/kg lipid). The mice were euthanized at 24 h and liposomal lipid in the peritoneal cavity was recovered and measured as under Materials and Methods. Values shown represent the mean from at least four animals ± SD.

1% of injected dose in the absence of tumor to almost 17% in the presence of tumor. Of these retained LUVs, 28% could be pelleted and, in contrast to studies in the absence of tumor, flow cytometric analysis suggested that the pelleted LUVs were primarily associated with peritoneal cells. Under conditions where targeting could be achieved (i.e., PEG-SA-LUVs injected ip into mice preinjected ip with BAH-MLVs) 42% of the injected dose was retained in the peritoneal cavity in the presence of tumor, comparable to that observed in the absence of tumor (35%). Remarkably, more than 90% of the retained PEG-SA-LUVs could be pelleted by centrifugation. The amount pelleted (1.25 μmol) is equivalent to the maximum amount of PEG-SA-LUVs that could be bound to 20 μmol of BAH-MLVs (based on the *in vitro* data shown in Fig. 2).

Other important conclusions that can be made from the data in Table 3 concern the studies on targeting of iv-administered PEG-SA-LUVs to BAH-MLVs present in the peritoneal cavity (see groups C and D, Table 3). In the absence of the P388 tumor approximately 27 nmol lipid were recovered in the peritoneal cavity 24 h after iv administration. This is equivalent to 0.82% of the injected lipid dose, a value that is significantly greater than that achieved in animals that have not received an MLV injection (results not shown). A 4- to 5-fold increase in liposome accumulation was observed in the presence of the tumor. Tumor-induced increases in peritoneal cavity accumulation are consistent with previous studies (20); however, the values obtained are significantly less than the 10- to 20-fold increases achieved using tumor bearing animals that were not given ip injections of MLVs. This effect appears to be a consequence of MLV injection-induced increases in accumulation of iv-injected LUVs (data not shown) rather than a MLV-mediated reduction in the tendency of ascitic tumors to promote extravasation.

Perhaps the most important result summarized in Table 3 is that in the presence and absence of tumor, preinjection of BAH-MLVs (in comparison with control MLVs) did not promote delivery of iv-injected PEG-SA-LUVs to the peritoneal cavity. There was, however, approximately a 6-fold increase in the amount of pelleted PEG-SA-LUVs when BAH-MLVs were present in the cavity. This strongly suggests that the presence of a target population in an extravascular site will not increase passive accumulation within the region. However, regionally localized liposomes with surface-associated target-specific ligands will bind to a target after extravasation. The efficiency of targeting achieved under the conditions employed here was adequate with 55 to 65% of the regionally localized liposomes copelleting with the target MLV populations. Relative to controls, however, the maximum increase in target-specific delivery, as determined by the simple centrifugation technique employed here, was about 6-fold.

An additional approach testing whether PEG-SA-LUVs can be targeted *in vivo* to regionally localized BAH-MLVs concerned evaluating delivery of liposomal lipid to solid tumors derived following sc injection of Lewis lung sarcoma cells. Two tumors were established per mouse, ranging in size from 200 to 500 mg. One tumor was given an intratumoral injection of BAH-MLVs while the contralateral tumor was injected with

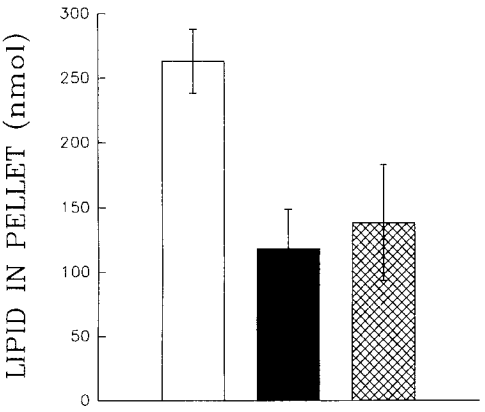

FIG. 5. Targeting of PEG-SA-LUVs (injected iv) to BAH-MLVs at a subcutaneous site. Mice were injected subcutaneously in the right and left thighs with 3×10^5 Lewis lung cells and allowed to grow 10–13 days, at which time BAH-MLVs and MLVs (20 μmol lipid) were injected subcutaneously into the right and left tumor sites, respectively. One hour later, animals were injected (iv) with PEG-SA-LUV or LUV (3.29 μmol lipid/mouse; 100 mg/kg lipid). Combinations included BAH-MLVs and PEG-SA-LUVs (□), MLVs and PEG-SA-LUVs (■), and BAH-MLVs and LUVs (▨). Twenty-four hours later the animals were euthanized and lipid levels at the subcutaneous tumor sites were determined as described under Materials and Methods. Values shown represent the mean from at least four animals ± SE of the mean.

control MLVs. One hour after injection of MLVs the animals were given an iv injection of either PEG-SA-LUVs or control LUVs. One day later tumors were removed and the amount of LUV lipid per gram of tumor was determined. In contrast to the peritoneal model summarized in Table 2, the results (shown in Fig. 5) clearly demonstrate that the presence of BAH-MLVs within the solid tumor promoted delivery of PEG-SA-LUVs. Compared to controls, consisting of tumors injected with control MLVs or animals treated with protein-free LUVs, there was at least a 2-fold increase in tumor delivery when the targeting reaction between LUVs and MLVs was facilitated by streptavidin and biotin.

DISCUSSION

Previous studies from this laboratory have demonstrated that doxorubicin-loaded PEG-SA-LUV injected iv can be efficiently targeted to cells prelabeled with a biotinylated antibody (6). Through the use of RES blockade and incorporation of PEG-modified lipids, PEG-SA-LUVs exhibit enhanced circulation lifetimes and an increased tendency to escape the blood compartment, resulting in increased accumulation of PEG-SA-LUVs within a potential target site. Further optimization of this targeting approach, however, requires a better understanding of how target variables such as target antigen number and target cell number influence PEG-SA-LUVs delivery and localization within a target site. For this reason we have developed a model target system based on the high-affinity binding reaction between PEG-SA-LUVs and biotin incorporated on the surface of a large multilamellar vesicle. Initial studies characterizing this model system have been described in this report and the results obtained lead to several general conclusions regarding the potential for targeting liposomal drug carriers to extravascular sites.

The model approach described here used PEG-SA-LUVs as the targeted drug carrier and biotin-labeled MLVs as the target. Studies describing the optimization of PEG-SA-LUVs for systemic delivery of anticancer drugs have been published elsewhere (6) and concluded that maintenance of liposome size and drug retention characteristics was required for optimal target-specific delivery. As demonstrated here (see Table 2) and elsewhere (30) large (>2 μm) multilamellar liposomes are retained well within the injection site after regional administration. The target MLVs were optimized for binding efficiency through the selection of appropriate biotinylated lipids incorporated in the MLVs. The studies here (see Fig. 1) demonstrate that biotin, linked to a saturated phospholipid via a six-carbon spacer arm, is optimal in terms of PEG-SA-LUV binding to MLVs. Further, the binding reaction was shown to be specific (Fig. 3) and was not influenced by prior *in vivo* exposure (Fig. 4 and Table 2).

The *in vivo* targeting studies based on PEG-SA-LUV binding to BAH-MLVs demonstrate that liposome targeting can be achieved. As expected, the most efficient target-specific delivery was achieved following ip injection of PEG-SA-LUVs in animals with BAH-MLVs localized in the peritoneal cavity. The results of the ip/ip targeting studies demonstrate that targeting efficiencies of greater than 98% can be obtained. *In vitro* studies, for example, suggest that a maximum of 1.3 μmol PEG-SA-LUV lipid can bind per 20 μmol BAH-MLV lipid. In the *in vivo* studies in the presence of tumor, the amount of pelleted PEG-SA-LUV was 1.25 μmol (or about 40% of the injected dose) in animals given an ip injection of 20 μmol BAH-MLVs (Table 3).

Clearly, the most challenging approach to *in vivo* targeting is that based on iv-administered PEG-SA-LUV that must access a target outside the vascular compartment. The model approach described here provides an ideal way in which to evaluate this approach. Under the conditions described here, the number of targets, BAH-MLVs, within the peritoneal cavity can be estimated at between 1×10^9 and 30×10^9 assuming that MLVs exhibit mean diameters in the range of 2 to 10 μm. Further, each BAH-MLV target has 10,000 to 200,000 target ligands expressed on the surface (calculation based on MLVs incorporating 1 mol% BAH-DSPE and having 5% of the lipid in the outer lamellae). Finally, the binding reaction between the target and the carrier is mediated by the high-affinity binding be-

tween biotin and streptavidin. Using this model, targeted PEG-SA-LUVs demonstrated at least a 6-fold increase in specific binding to BAH-MLVs compared to nontargeted LUVs, with as much as 90% of the available PEG-SA-LUVs bound to the target. However, the studies described here clearly indicate that having an excess of high-affinity target sites present in the peritoneal cavity does not promote accumulation of PEG-SA-LUVs following iv administration, even under conditions where the vascular permeability of the blood vessels lining the peritoneum has been increased in response to factors released from a growing ascitic tumor. In contrast, a twofold increase in accumulation of PEG-SA-LUVs was achieved within solid tumors preinjected with BAH-MLVs. Differences between the results obtained with the solid tumor and the peritoneal cavity may be due to the mechanisms governing liposome extravasation in these two models. In particular, blood vessels responsible for mediating movement of liposomes from the blood compartment to extravascular sites would be expected to be different in solid tumors that elicit vascularization through release of angiogenic factors (31) when compared to an ascitic tumor that releases factors that promote increased vascular permeability in preexisting blood vessels (32, 33).

In conclusion, the results presented in this study demonstrate that PEG-SA-LUVs can be targeted to MLVs incorporating a biotin-labeled phospholipid in an extravascular site. Saturated biotin-labeled phospholipids containing a linker arm to connect the biotin to the phospholipid yielded the most efficient binding of PEG-SA-LUVs to target MLVs *in vitro*. PEG-SA-LUVs incubated *in vitro* with normal mouse serum or recovered in the plasma of mice maintained their ability to bind to BAH-MLVs. Binding of PEG-SA-LUVs to BAH-MLVs in the peritoneal cavity was increased 6-fold and 17-fold for PEG-SA-LUVs injected iv and ip, respectively.

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