

# Accumulation of Protein-Coated Liposomes in an Extravascular Site: Influence of Increasing Carrier Circulation Lifetimes<sup>1</sup>

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## ABSTRACT

The primary objective of this work was to test whether increased blood levels and circulation lifetimes result in increased passive targeting of protein-coated liposomal drug carriers. The system used to evaluate this was based on i.v. injection of 100 nm of distearoyl phosphatidylcholine/cholesterol liposomes with covalently bound streptavidin. The circulation lifetime of these liposomes was increased by procedures that involved blockade of liposome uptake by phagocytic cells in the liver and/or the incorporation of a poly(ethylene glycol)-modified phospholipid [poly(ethylene glycol)<sub>2000</sub>-modified distearoyl phosphatidylethanolamine]. Blockade of liver phagocytic cells with a low predose (2 mg/kg of drug) of liposomal doxorubicin increased the circulation half-life of the streptavidin liposomes from less than 1 hr to greater than 3 hr. A further 2-fold increase

in circulating half-life (to approximately 7.5 hr) was achieved by using liposomes with 2 mole % of poly(ethylene glycol)<sub>2000</sub>-modified phosphatidylethanolamine. In combination with RES blockade, the circulation lifetimes of poly(ethylene glycol)-phosphatidylethanolamine containing streptavidin liposomes could be increased to greater than 12 hr. The ability of these liposomes to move from the plasma compartment to an extravascular compartment was measured by using the peritoneal cavity as a convenient, accessible, extravascular site. The tendency for liposomes to accumulate in this site was not, however, clearly dependent on circulating blood levels. Comparable levels of liposomes in the peritoneal cavity were achieved when using systems that exhibited significantly different circulation lifetimes.

Targeting liposomes to specific cells or tissues *in vivo* has been a major objective of research aimed at improving the therapeutic index of liposomal anticancer drugs (Straubinger *et al.*, 1988; Bankert *et al.*, 1989; Nässander *et al.*, 1992; Ahmad *et al.*, 1993). Liposome characteristics that must be optimized for *in vivo* targeting include target site specificity, binding affinity and, most importantly, physical access to the target cell population. Studies using monoclonal antibody-based drug carrier systems suggest that extravasation, the process whereby material within the blood compartment gains access to an extravascular site, will be dependent on several factors including vascular structure (Jain, 1990; Kohn *et al.*, 1992; Badger *et al.*, 1985), antibody circulation lifetimes (Rostaing-Capaillon and Casellas, 1990) as well as antigen expressed on tumor cells (Thomas *et al.*, 1989; Sung

*et al.*, 1992). Not unexpectedly, the rate of transport of macromolecules from the blood compartment to an extravascular site is dependent on size (Nagy *et al.*, 1989).

It is now well documented that liposomes, with no specific surface-associated targeting information, can access extravascular sites in disease tissues such as tumors (Papahadjopoulos *et al.*, 1991; Bally *et al.*, 1994). This passive targeting process is known to be dependent on liposome size and circulation longevity (Proffitt *et al.*, 1983; Gabizon and Papahadjopoulos, 1988; Gabizon, 1992). It can be suggested on the basis of these data that any liposomal carrier formulated for *in vivo* ligand-mediated targeting applications would have to exhibit characteristics of uniform small size and enhanced circulation lifetime. Limitations of liposome based targeted delivery systems include the fact that procedures used to attach targeting ligands to the liposome surface promote aggregation (Loughrey *et al.*, 1990b, 1993) and that the targeting ligand itself can result in immune recognition of the carrier (Longman *et al.*, 1995; Aragnol and Leserman, 1986). Both of these attributes promote clearance of i.v. adminis-

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**ABBREVIATIONS:** RES, reticuloendothelial system; PEG, poly(ethylene glycol); PE, phosphatidylethanolamine; SA-LUV, streptavidin liposomes; DSPC, distearoyl phosphatidylcholine; SPDP, N-succinimidyl 3-(2-pyridyl)dithio) propionic acid; Chol, cholesterol; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, N-ethylmaleimide; MPB-DPPE, N-(4-(P-maleimidophey)butyryl) dipalmitoyl-phosphatidylethanolamine; LUV, large unilamellar vesicles; PEG-PE, poly(ethylene glycol)-modified phosphatidylethanolamine; HBS, 150 mM NaCl and 25 mM HEPES; QELS, quasielastic light scattering; PBS, phosphate-buffered saline.

tered targeted liposomes. It is well established, for example, that aggregated liposomes formed during coupling reactions used to attach targeting proteins to liposomes are removed rapidly after i.v. administration (Loughrey *et al.*, 1990b, 1993). This clearance is mediated, in part, by phagocytic cells of the RES. Furthermore, when the targeting ligand used is a whole antibody, the presence of surface-associated Fc portions may further increase liposome clearance (Aragno and Leserman, 1986).

Phagocytic cells of the RES are known to be responsible for recognition and removal of foreign particles, including liposomes (Proffitt *et al.*, 1983; Allen *et al.*, 1989; Papahadjopoulos *et al.*, 1991). Strategies, therefore, have been developed to limit RES uptake of targeted liposomes. One approach involves incorporation of lipids, such as the ganglioside  $G_{M1}$  or PEG-modified phospholipids, which are known to increase the circulation lifetime of liposomes (Allen *et al.*, 1989; Papahadjopoulos *et al.*, 1991). However, it is apparent that a balance must be struck between RES avoidance and target specific binding, because incorporation of lipids such as PEG-modified PEs can interfere with binding (Klibanov *et al.*, 1991). A second, more invasive, procedure involves the use of agents that specifically "block" phagocytic cells of the liver, a primary site of accumulation after i.v. administration of liposomes (Proffitt *et al.*, 1983; Bally *et al.*, 1990). Previous studies from this laboratory, for example, have demonstrated that pretreating mice with low dose liposomal doxorubicin results in a 90% reduction in liver accumulation of subsequently administered (i.v.) liposomes (Bally *et al.*, 1990). Significant increases in blood levels of liposomal lipid are observed in these pretreated animals.

This laboratory has been pursuing an *in vivo* liposome targeting approach that involves binding SA-LUV to target cells prelabeled with biotinylated antibody (Loughrey *et al.*, 1990a, 1993; Longman *et al.*, 1995). Results have shown that this two-step targeting procedure was as efficient as approaches involving antibody-coated liposomes when labeling cells *in vitro* (Longman *et al.*, 1995). However, *in vivo* targeting of these liposomes was not efficient, despite using conditions in which the target cell population was labeled extensively with a surface-associated biotinylated antibody. A major limitation concerned the inability of these carriers to access the target cell population after i.v. administration. The studies presented here address this problem and examine the use of RES blockade and/or incorporation of PEG-modified lipids to maximize the circulation lifetimes of SA-LUV and thus increase the level of liposomes achieved in an extravascular site. Substantial increases in circulation lifetimes were attained; however, the tendency for the liposomes to escape the blood compartment could not be directly correlated to increased circulation lifetimes.

## Materials and Methods

**Materials.** DSPC was obtained from Avanti Polar Lipids and SPDP was from Molecular Probes (Eugene, OR). Biotinylated anti-mouse Thy 1.2 antibody was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Chol, DTT,  $\beta$ -mercaptoethanol, HEPES, NEM, streptavidin, Sephadex G-50, D-biotin, sepharose CL-4B and all salts were obtained from Sigma Chemical Co. (St. Louis, MO). [ $^3$ H]Cholesteryl hexadecyl ether was obtained from New England Nuclear (Boston, MA) and [ $^{14}$ C]biotin was from Amersham (Des Plaines, IL). MPB-DPPE and PEG-PE were prepared by Dr. S.

Ansell and generously donated by Northern Lipids Inc. (Vancouver, Canada).

**Preparation of liposomes.** LUV were prepared using the extrusion method described by Hope *et al.* (1985). Briefly, lipid films ( $\times$  mole % of PEG<sub>2000</sub>-DSPE, 54- $\times$  mole % of DSPC, 45 mole % of Chol and 1 mole % of MPB-PE) were prepared from a chloroform solution by drying under a stream of nitrogen followed by vacuum evaporation for 2 hr. Lipids were then hydrated at 65°C in 150 mM NaCl and 25 mM HEPES, pH 7.5 (HBS) by vortex mixing such that a final lipid concentration of 50 mM was achieved. LUV were then prepared by extrusion (5 times) at 65°C through stacked polycarbonate filters (100 nm pore size) (Nucleopore, Inc., Pleasanton, CA) using an Extruder (Lipex Biomembranes, Vancouver, Canada). LUV were subjected to five freeze-thaw cycles, followed by repeated extrusion (5 times). Mean vesicle diameters were determined by QELS by using a Nicomp 270 submicron particle sizer operating at a wavelength of 632.8 nm. Typically, liposomes were prepared such that the nonexchangeable, nonmetabolizable lipid [ $^3$ H]Cholesteryl hexadecyl ether 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 procedures (Loughrey *et al.*, 1990b). 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 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 used immediately for coupling experiments. The extent of modification of streptavidin was determined by estimating the concentration of the protein at 280 nm (molar extinction coefficient,  $E_{280}$ :  $1.66 \times 10^5$ ) before the addition of DTT, and the 2-thiopyridine concentration was measured at 343 nm ( $E_{343}$ : 7550) 10 min after addition of DTT, according to published procedures (Carlsson *et al.*, 1978).

**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.* (1990b). Briefly, SPDP-streptavidin was incubated with liposomes at a ratio of 75  $\mu$ g of protein per  $\mu$ mol of lipid (10 mM final lipid concentration). The coupling reaction was stopped after the desired incubation time by the addition of DTT (10 times molar excess over MPB-PE) followed 2 min later by the addition of NEM (10 times molar excess over DTT + SPDP), and unassociated protein was removed by gel filtration on sepharose CL-4B equilibrated with HBS. In the absence of PEG-PE incorporation, cross-linking of vesicles during coupling resulted in liposome aggregation (300–400 nm in diameter as determined by QELS). Where indicated, 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) (Loughrey *et al.*, 1990b). The extent of streptavidin coupling to liposomes was determined by using a functional assay that measured binding of [ $^{14}$ C]biotin to streptavidin. Briefly, SA-LUV (0.50  $\mu$ mol of 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.

***In vitro* targeting of SA-LUV to P388 cells.** The murine lymphocytic leukemia cell line P388 was obtained from NCI Tumor Repository (Bethesda, MD) and grown in Roswell Park Memorial Institute 1640 (Flow Laboratories, Rockville, MD) supplemented with 10% fetal calf serum (Flow Laboratories). P388 cells grown in culture (37°C in 5% CO<sub>2</sub>) were aliquoted (10<sup>7</sup> cells/ml) into conical bottomed polypropylene tubes and incubated with biotinylated anti-mouse Thy 1.2 antibody or alone in PBS containing 2% bovine serum albumin (w/v) for 30 min at 4°C. The final antibody concentration for

these studies was 12.5 nM. After washing twice with PBS, cells were incubated with SA-LUV containing various mole percentages of PEG-PE (2 mM final liposomal lipid concentration) for 30 min at 4°C. The cells were then washed 3 times with PBS and cell-associated lipid was assayed. Controls included cells incubated with liposomes not conjugated with streptavidin.

**Animals.** Female CD1 mice (20–25 g) and female BDF1 mice (18–22 g) were obtained from Charles River Canada Inc. (St-Constant, Quebec, Canada). Groups of four mice per experimental point were given the specified treatment in a single i.v. dose via the lateral tail vein. The dose was administered in a volume of 200  $\mu$ l. Blood was collected after carbon dioxide asphyxiation via cardiac puncture and placed in EDTA-treated microtainers (Becton-Dickinson, Canada, via VWR Scientific, Edmonton, Alberta). Plasma was prepared by centrifuging (200  $\times$  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.

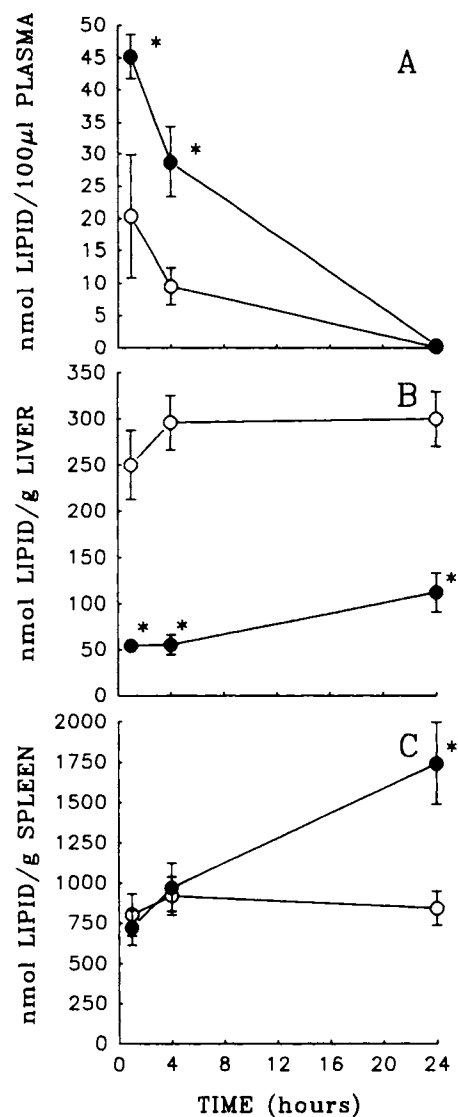
Biodistribution studies were performed on the same mice used for plasma clearance studies. After the animals were terminated, peritoneal cavities were lavaged with 5 ml of indicator free and  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  free Hanks media that was injected i.p. The abdomen was gently massaged and the peritoneal fluid was removed with a syringe equipped with a 22-g needle. Peritoneal fluid was assayed for liposomal lipid as indicated below. BDF1 mice bearing P388 tumors were prepared by inoculating mice i.p. with  $1 \times 10^6$  P388 cells. Tumors progressed for 4 days before i.v. injection of the specified liposomal preparation. Four days after cell administration, there was no measurable ascites fluid. Lavage fluid from either tumor-bearing or tumor-free animals that contained red cell contamination was discarded.

After peritoneal lavage, the liver and spleen were removed from each animal and weighed. Organs were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in distilled  $\text{H}_2\text{O}$  to produce a 10% (w/v) homogenate for the spleen and 20% (w/v) homogenate for the liver. Tissue homogenates (200  $\mu$ l for spleen, 100  $\mu$ l for liver) were digested with 500  $\mu$ l of Solvable (DuPont Canada, Inc., Mississauga, Ontario, Canada) for 3 hr at 50°C. Samples were allowed to cool to room temperature and 50  $\mu$ l of 200 mM EDTA was added to prevent foaming during decolorizing with 200  $\mu$ l of 30% hydrogen peroxide. Twenty-five microliters of 10 N HCl were added to reduce chemiluminescence. Subsequently, samples were mixed with 5 ml of Ultima Gold scintillation cocktail (Packard, Mississauga, Ontario, Canada) before counting on a Packard 1900 TR scintillation counter.

**Statistical analysis.** The statistical test used to compare groups was analysis of variance from the software package Statistica by StatSoft (Tulsa, OK). Specifically, the Scheffé Test within the Post Hoc Comparison of Means was used to compare different groups against each other.

## Results

**Influence of RES blockade on plasma clearance, liver uptake and peritoneal cavity accumulation of SAV-LUV.** The studies reported here investigate two procedures to increase the passive targeting of protein-coated liposomes, one based on inhibiting liposome uptake by phagocytic cells of the liver and the second based on incorporation of PEG-modified phospholipids. It has been shown that uptake of liposomes by cells within the liver can be significantly reduced by pretreating animals with a low dose of liposomal doxorubicin (2 mg of drug/kg) (Bally *et al.*, 1990). This liver blockade is thought to be mediated by accumulation of the cytotoxic drug doxorubicin in liver K upffer cells. Results in figure 1 illustrate how liver blockade promotes increased circulation lifetime and inhibits liver uptake of SA-LUV. In



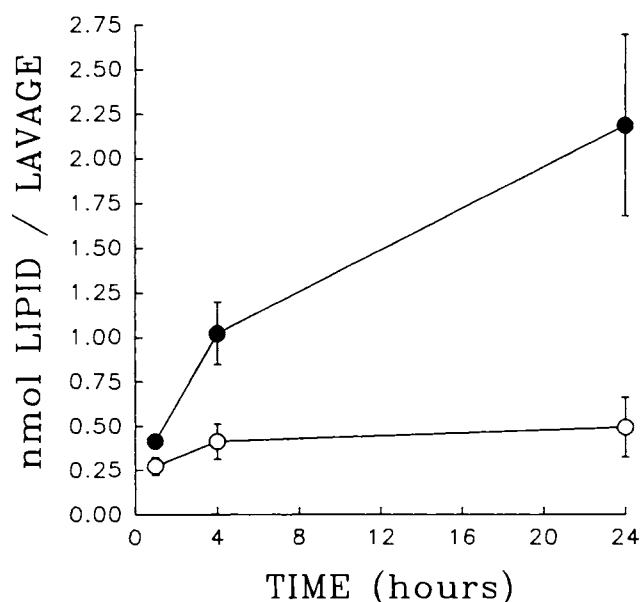
**Fig. 1.** Biodistribution of SA-LUV after liver blockade. Twenty-four hours after injection of a predose of liposomal doxorubicin [10 mg/kg of lipid, drug/lipid ratio of 0.2 (mol/mol)] (●) or empty liposomes (○), SA-LUV were injected via a lateral tail vein at a dose of 0.66  $\mu$ mol of lipid per mouse (20 mg/kg of lipid). The mice were terminated at the indicated times and the level of SA-LUV lipid was determined (see "Materials and Methods") for the plasma (A), liver (B) and spleen (C). Values shown represent the mean of results from at least four animals  $\pm$  S.E. of the mean. Asterisks indicate data points that were significantly different (\* $P < .05$ ).

this study, liposomes with 44  $\mu$ g of streptavidin per  $\mu$ mol of lipid, exhibiting a mean diameter of 160 nm (achieved after extrusion of cross-linked protein-liposome aggregates), were administered at a lipid dose of 20 mg/kg (0.66  $\mu$ mol of lipid per mouse). Mice were pretreated with either DSPC/Chol "empty" liposomes (10 mg/kg of lipid) or DSPC/Chol liposomal doxorubicin (10 mg/kg of lipid; 2 mg/kg of drug). These groups are referred to herein as control and pretreated animals, respectively. At this low lipid predose, "control" animals yielded results identical to animals receiving a predose of saline (results not shown). Four hours after administration of SA-LUV, there was greater than 3 times the level of liposomal lipid in the plasma of pretreated animals (40% of injected dose) (fig. 1A). At this same time point, there was a

5-fold reduction in the amount of lipid recovered in the liver of pretreated animals (11% of injected dose compared to 52% for control animals) (fig. 1B). Consistent with previous results (Bally *et al.*, 1990), accumulation of SA-LUV in the spleen of pretreated animals was significantly greater than that obtained for controls (27 and 13% of injected dose, respectively) (fig. 1C).

In addition to measuring plasma levels of SA-LUV, the tendency of these liposomes to leave the blood compartment and enter an extravascular site was assessed by measuring the time-dependent accumulation of liposomal lipid in the peritoneal cavity. The vascular structure lining the peritoneal cavity of control animals has been shown to be relatively impermeable to circulating macromolecules, therefore movement of liposomes into this compartment represents a stringent test of extravasation (Flessner *et al.*, 1985). Transport of macromolecules (fluorescently labeled dextran) from the blood into the peritoneal cavity in mice has been shown to be dependent on macromolecular size and circulating blood levels (Nagy *et al.*, 1989). The influence of pretreatment with liposomal doxorubicin (to increase circulation blood levels) on the transfer of SA-LUV from the blood compartment to the peritoneal cavity is shown in figure 2. Liposomal lipid, as measured by using the nonexchangeable, nonmetabolizable lipid label ( $^3\text{H}$ )cholesteryl hexadecyl ether) (Scherphof *et al.*, 1987), could be recovered from the peritoneal cavity by lavage of animals given (i.v.) SA-LUV. The level of lipid recovered was 2 to 5 times greater in pretreated animals than in controls. However, even in pretreated animals, the level of liposomal lipid in the peritoneal cavity was only 0.35% of injected dose.

**Characterization of protein coupling, plasma clearance, liver accumulation, target cell binding and extravasation of SA-LUV prepared with PEG-modified phospholipid.** The second, less invasive, approach used to



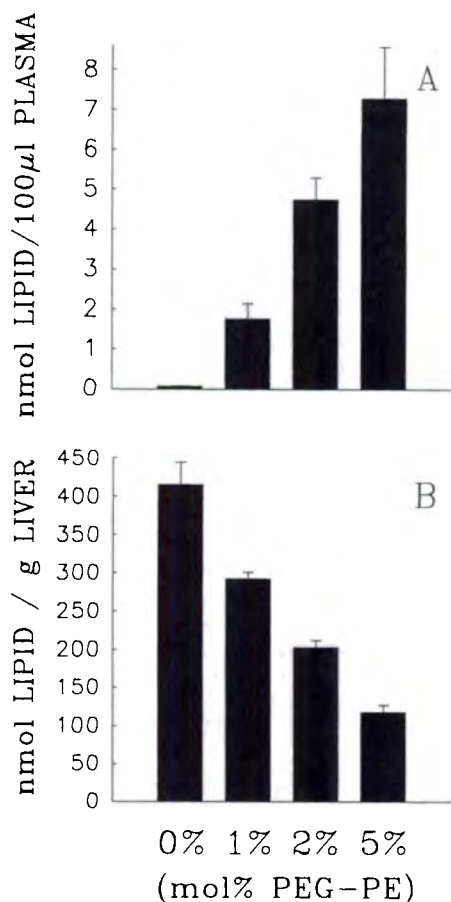
**Fig. 2.** Accumulation of SA-LUV in the peritoneal cavity after liver blockade. Predoses of liposomal doxorubicin (●) and empty liposomes (○) and the subsequent SA-LUV dose were as described in figure 1. The group predosed with liposomal doxorubicin was significantly different from control ( $P < .05$ ) at all time points. Values shown represent the mean of results from at least four animals  $\pm$  S.E. of the mean.

increase the circulation lifetimes of SA-LUV involved incorporation of PEG-modified phospholipids. It is well established that PEG-modified lipids, when incorporated into protein-free liposomes, dramatically increase circulation lifetimes (Papahadjopoulos *et al.*, 1991; Allen *et al.*, 1991; Mayhew *et al.*, 1992). Preliminary data from other laboratories suggest that incorporation of PEG-modified lipids may, however, interfere with protein coupling reactions (Klibanov *et al.*, 1991; Harasym *et al.*, 1995). Therefore, preliminary studies summarized here assessed how incorporation of PEG<sub>2000</sub>-DSPE at various mole percentages in LUV used for protein coupling influenced coupling rates and target cell binding. Initial studies focused on defining the reaction conditions required to achieve 35 to 45  $\mu\text{g}$  of streptavidin per  $\mu\text{mol}$  of lipid. Protein coupling rates were significantly reduced when using liposomes with 5 mole % of PEG-PE (data not shown). However, satisfactory (35–45  $\mu\text{g}$ ) levels of coupling could be achieved with longer incubation times.

As demonstrated elsewhere (Harasym *et al.*, 1995), the liposome aggregation reaction, typically associated with the coupling procedures used here, was eliminated when using liposomes with 2 and 5 mole % of PEG-PE. Even at values as low as 1 mole %, there was a significant reduction in the tendency of these liposomes to aggregate during protein coupling. Because it is well established that decreases in liposome size promote increased circulation lifetimes, it was expected that incorporation of PEG-PE would also significantly increase retention of SA-LUV in the plasma compartment. As shown in figure 3A, the level of SA-LUV in plasma 24 hr after i.v. administration increases with increasing PEG-PE levels. Differences in plasma levels observed for SA-LUV with 0, 1 and 2 mole % of PEG-PE can be attributed to changes in liposome size. The size of SA-LUV with 2 and 5 mole % of PEG-PE (120 nm diameter) were, however, comparable as judged by QELS measurements. Therefore, increased plasma levels observed for liposomes with 5 mole % of PEG-PE (fig. 3B) are likely due to a direct effect of this lipid on reducing RES accumulation (Papahadjopoulos *et al.*, 1991; Mayhew *et al.*, 1992).

The effect of various mole % of PEG-PE on the binding of SA-LUV to P388 cells prelabeled with a biotinylated antibody is shown in figure 4. These results were obtained by using a two-step targeting approach (Loughrey *et al.*, 1993; Longman *et al.*, 1995) in which P388 cells were first incubated (4°C, 30 min) with or without biotinylated anti-Thy 1.2 antibodies. After extensive washing, the cells were resuspended in media and SA-LUV were added. Binding of liposomal lipid to these cells was determined after a 30-min incubation and extensive washing, as described under "Materials and Methods." The data demonstrate that incorporation of 5 mole % of PEG-PE significantly ( $P < .005$ ) reduced SA-LUV association with P388 cells prelabeled with biotinylated anti-Thy 1.2 Ab.

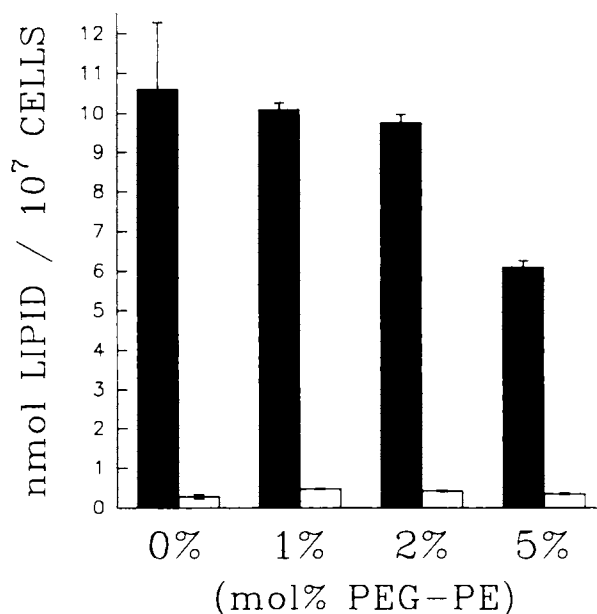
**Maximizing circulation lifetimes of SA-LUV by combining RES blockade and incorporation of PEG-modified lipids.** The characterization studies summarized above suggest that incorporation of 2 mole % of PEG<sub>2000</sub>-DSPE (52 mole % of DSPC, 45 mole % of Chol and 1 mole % of MPB-PE) in liposomes used for covalent attachment of streptavidin was optimal with respect to achieving efficient protein coupling with no increase in vesicle size and no reduction in target cell association as mediated through binding to a biotinylated antibody. By using this liposomal formulation,



**Fig. 3.** Biodistribution of SA-LUV incorporating various mole % of PEG<sub>2000</sub>-DSPE. SA-LUV containing PEG<sub>2000</sub>-DSPE at 0, 1, 2 and 5 mole % (180, 140, 120 and 120 nm in size, respectively) were injected via a lateral tail vein at a dose of 0.66 µmol of lipid per mouse (20 mg/kg of lipid). The mice were sacrificed at 24 hr and lipid levels were determined for the tissues indicated. All groups were significantly different ( $P < .05$ ) from each other. Values shown represent the mean of results from at least four animals  $\pm$  S.E. of the mean.

studies combining the influence of PEG-PE incorporation and blockade of liver phagocytic cells were initiated. The results, shown in figure 5, indicate that a combination of these strategies resulted in remarkably improved circulation lifetimes (fig. 5A) and significant reductions in liver accumulation (fig. 5B). At 24 hr, for example, there were 15 times more liposomal lipid present in the plasma of mice given SA-LUV, incorporating 2 mole % of PEG-PE when those mice were predosed with liposomal doxorubicin (2 mg/kg of drug) compared to liposomes incorporating 0 mole % of PEG-PE in control mice (11.2% vs. 0.67% of injected dose, respectively). Furthermore, the lowest level of lipid accumulation in liver (less than 6% of the injected dose at 24 hr) was achieved when using 2 mole % of PEG-PE containing SA-LUV in combination with blockade of liver phagocytes.

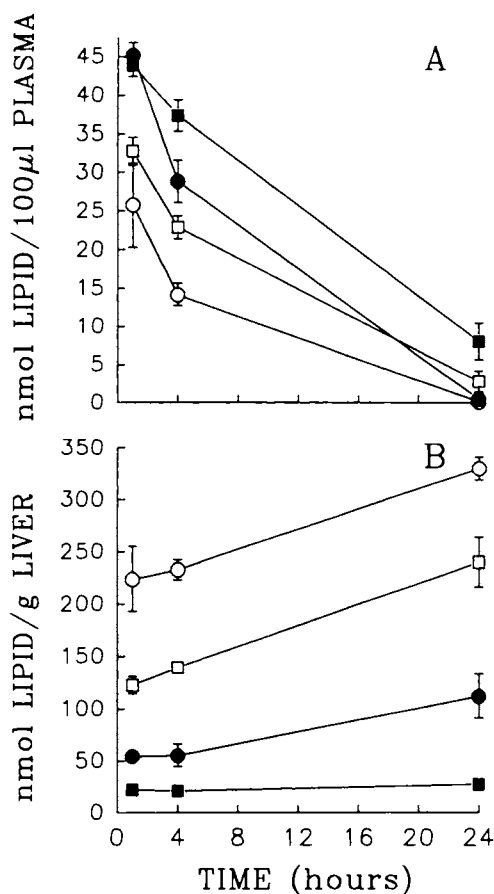
The impact of combining both RES blockade and incorporation of PEG-PE on the level of liposomal lipid accumulation within the peritoneal cavity 24 hr after i.v. administration is shown in figure 6. The results demonstrate that the greatest effect on liposome movement from the plasma compartment to this extravascular site occurs only as a result of blockade of liver phagocytes. In both the presence and absence of 2 mole % of PEG-PE, there was a 4- to 5-fold increase in



**Fig. 4.** Quantification of cell-associated lipid after targeting of SA-LUV incorporating various mole % of PEG<sub>2000</sub>-DSPE to P388 cells *in vitro*. SA-LUV (35–45 µg of streptavidin per µmol of lipid) incorporating PEG<sub>2000</sub>-DSPE were prepared as described under "Materials and Methods." P388 cells (10<sup>7</sup>) were incubated with (solid bars) or without (empty bars) biotinylated antimouse Thy 1.2 Ab (10 µg) for 30 min at 4°C. Cells were washed with PBS before the addition of SA-LUV (2 µmol of lipid, 1 ml volume, 2 mM final concentration) with 0, 1, 2 and 5 mole % of PEG<sub>2000</sub>-DSPE. After a 30-min incubation at 4°C, the cells were washed and cell associated lipid was determined as detailed under "Materials and Methods." All groups incubated with antibody were significantly different ( $P < .001$ ) from groups incubated without antibody. SA-LUV with 5 mole % of PEG-PE (+Ab) were significantly different ( $P < .005$ ) from all other SA-LUV (+Ab).

delivery of SA-LUV to this site when animals were pretreated with liposomal doxorubicin (0.35% of injected dose compared to 0.07% for control animals).

**Transfer of SA-LUV from the plasma compartment to the peritoneal cavity of mice with established peritoneal tumors.** The tendency of SA-LUV to exit the blood compartment was evaluated in mice bearing P388 cells grown in the peritoneal cavity of BDF1 mice. The results of this study, in which SA-LUV were given (i.v.) to BDF1 mice with or without a tumor burden (see "Materials and Methods") are shown in figure 7. Figure 7 illustrates three important points. First, the plasma levels of SA-LUV with 2 mole % of PEG-PE obtained at 24 hr after i.v. administration are reduced in mice with P388 tumors (fig. 7A). This effect is most significant under conditions in which uptake by phagocytic cells of the liver was reduced through pretreatment with low dose liposomal doxorubicin (filled bars) ( $P < .05$ ). Such data are consistent with results from other laboratories, which suggest that tumor cells secrete factors that increase vascular permeability to macromolecules within blood vessels (Kohn *et al.*, 1992; Senger *et al.*, 1983). Second, the presence of the peritoneal tumor had no impact on the liver accumulation of SA-LUV with 2 mole % of PEG-PE (fig. 7B). Consistent with results in figure 5, the level of liposomal lipid in the blood was increased and the level in the liver decreased when animals had been pretreated with a low dose liposomal doxorubicin. Third, the presence of tumor cells in the peritoneal cavity significantly increased the level of SA-LUV accu-

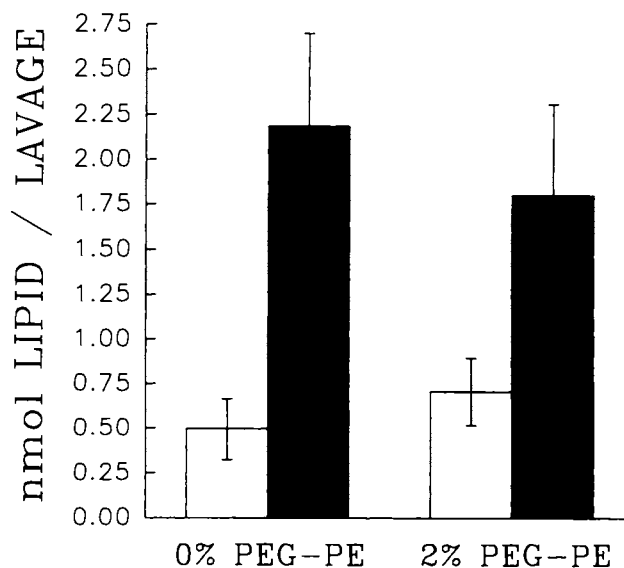


**Fig. 5.** Biodistribution of SA-LUV incorporating 2 mole % of PEG<sub>2000</sub>-DSPE after liver blockade. At 24 hr after injection of a low dose, liposomal doxorubicin [10 mg/kg of lipid, drug/lipid ratio of 0.2 (mole %)] (■ and ●) or saline (□ and ○), SA-LUV containing 0 mole % (●, with blockade; ○, without blockade) or 2 mole % (■, with blockade; □, without blockade) of PEG<sub>2000</sub>-DSPE were injected via a lateral tail vein at a dose of 0.66 µmol of lipid per mouse (20 mg/kg). The mice were sacrificed at the indicated times and lipid levels of this subsequent dose were determined for the tissues indicated. Values shown represent the mean from at least four animals ± S.E. of the mean. For plasma at 24 hr, the group treated with liposomal doxorubicin and SA-LUV containing 2 mole % of PEG-PE were significantly different ( $P < .05$ ) from all other groups. For liver, all points were significantly different ( $P < .05$ ) from each other.

mulation in the peritoneal cavity (fig. 7C) ( $P < .001$ ). There was a 50-fold increase in the amount of liposomal lipid recovered from the peritoneal cavity of mice bearing an established P388 tumor compared to nontumor-bearing animals. Surprisingly, the level obtained within this extravascular site, equivalent to 7% of the injected lipid dose 24 hr after i.v. administration, was similar in the presence or absence of liver blockade. Analogous to results obtained in the absence of tumor, the level achieved within this extravascular site at 24 hr was not necessarily related to levels of liposomal lipid found in the circulation.

### Discussion

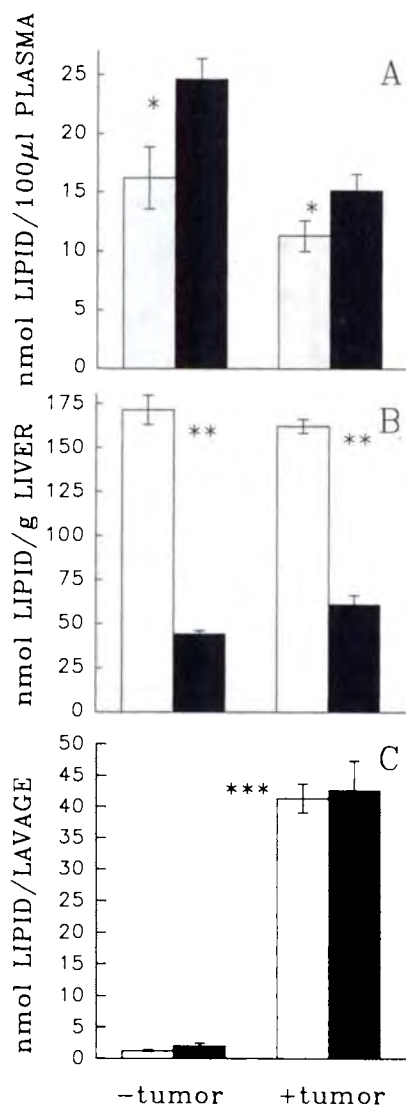
The studies presented in this report were aimed at optimizing the circulation lifetimes of SA-LUV in an attempt to increase the tendency of these liposomes to exit the blood compartment and access an extravascular site (passive targeting). It has been suggested that the natural tendency of



**Fig. 6.** Accumulation of SA-LUV incorporating 2 mole % of PEG<sub>2000</sub>-DSPE in the peritoneal cavity after liver blockade. Predoses (solid bars, with blockade; empty bars, without blockade) and subsequent doses of SA-LUV incorporating 0 or 2 mole % of PEG<sub>2000</sub>-DSPE were as described in figure 5. Data points with blockade were significantly different ( $P < .05$ ) from points without blockade.

liposomes to accumulate in sites of tumor growth is dependent on carriers exhibiting extended circulation lifetimes (Proffitt *et al.*, 1983; Gabizon and Papahadjopoulos, 1988; Gabizon, 1992). As demonstrated in this report, the circulation lifetime of liposomes can be increased by use of procedures that minimize liposome uptake by phagocytic cells of the RES. For example, SA-LUV prepared with 2 mole % of PEG-PE, a lipid known to engender long circulation lifetimes by minimizing RES uptake, exhibit increased circulation longevity (see fig. 3). Alternatively, preadministration of a low dose of liposomal doxorubicin (2 mg of drug/kg) blocks the ability of phagocytic cells in the liver to accumulate these protein-coated liposomes. It is demonstrated (see figs. 2 and 6) that these procedures increase the circulation lifetime of the carrier and improve delivery to a defined extravascular compartment (the peritoneal cavity). Passive targeting of the protein-coated liposomes, however, appears to be limited by factors in addition to circulation lifetime. In particular, mechanisms controlling the ability of liposomes to leave the blood and enter an extravascular site must be specifically identified.

It is believed that movement of liposomes from the blood compartment into tumors occurs as a consequence of tumor-mediated changes in blood vessel structure. Tumor blood vessels, for example, are more permeable to circulating macromolecules than comparable vessels in normal tissue. Leakage may occur through pores or fenestrations within the endothelial cells lining the blood vessels (Senger *et al.*, 1983; Jain, 1990; Kohn *et al.*, 1992). Alternatively, transport may involve transcytosis through endothelial cells via cytoplasmic vesicles (Senger *et al.*, 1983; Jain, 1990; Kohn *et al.*, 1992). From the data reported here, it is apparent that increased circulation lifetime, achieved through incorporation of PEG-PE, for example, does not necessarily promote extravasation to the peritoneal cavity. This is contrary to other reports in which prolonged circulation times of PEG-PE-containing li-



**Fig. 7.** Influence of liver blockade and presence of tumor on circulation lifetimes and accumulation in the liver and peritoneal cavity of SA-LUV given (i.v.). Three days after i.p. injection of  $10^6$  P388 cells or saline, animals were given (i.v.) a predose of liposomal doxorubicin [10 mg/kg of lipid, drug/lipid ratio of 0.2 (mole %)] (solid bars) or saline (empty bars). SA-LUV containing 2 mole % of PEG<sub>2000</sub>-DSPC were injected (i.v.) 24 hr later at a dose of 0.66  $\mu$ mol of lipid per mouse (20 mg/kg). The mice were sacrificed at 24 hr and lipid levels of the subsequent injection were determined for the blood, liver and peritoneal cavity, as described under "Materials and Methods." Values shown represent the mean of results from at least four animals  $\pm$  S.E. of the mean. Statistical significance was indicated as follows: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

posomes enhanced uptake by tumors (Gabizon and Papahadjopoulos, 1988; Yuan *et al.*, 1994). However, it can be argued that these comparisons between conventional liposomes and sterically stabilized liposomes were not particularly valid. As noted in these references, the studies were typically done under conditions in which the blood levels of conventional liposomes were significantly lower than those of the PEG-PE-containing liposomes. This is in contrast to the results reported here, in which blood levels were somewhat comparable (e.g., only a 2-fold difference in circulation half-life and blood levels achieved at 24 hr). It is interesting to note that the data presented suggest that the transport process is in effect saturable. Increases in plasma lipid levels conferred by

PEG-PE in addition to the effect of RES blockade, therefore, did not result in enhanced extravasation. This is a surprising conclusion that is inconsistent with the concept that extravasation occurs primarily through pores within tumor blood vessels.

Liposome transport mediated by pores or transcytosis processes will be dependent on particle size. Processes involving endocytosis, for example, will likely be restricted to particles exhibiting diameters of 100 nm or less (Machy *et al.*, 1982). It should be noted that liposome size is a characteristic that will influence both circulation lifetime and the potential for liposomes to access to an extravascular site (Lui *et al.*, 1992). With regards to preparation of protein-coated liposomes for targeting purposes, methods described here provide a means whereby liposome size can be easily controlled during the covalent attaching of targeting proteins. Coupling-induced increases in vesicle size has been identified as a significant problem associated with protocols relying on the use of heterobifunctional cross-linking agents. Incorporating small quantities (2 mole %) of PEG-modified PE completely prevented aggregation associated with coupling, but did not effect the rate of protein coupling or the extent to which the resulting liposomes could bind to target cells (fig. 4).

Combining the strategies of RES blockade and incorporation of PEG-PE further decreased liver uptake and increased circulation lifetimes of SA-LUV, but did not lead to increased passive accumulation of these carriers in the peritoneal cavity. There are at least two possible reasons for this lack of synergism between the two strategies. First, the mechanism by which liposomes exit the vasculature and enter the peritoneal cavity may be rate limiting. Two mechanisms have been proposed. Leakage may occur through pores or fenestrations within the endothelial cells lining the blood vessels, or may involve transcytosis through endothelial cells by way of cytoplasmic vesicles (Kohn *et al.*, 1992). Second, the manner in which liver blockade and PEG-PE increase blood circulation lifetimes of SA-LUV may influence the mechanism involved in passive targeting. Liver blockade directly inhibits the phagocytic capability of Kupffer cells, whereas PEG-PE bestows a hydrophilic coating and steric barrier on the liposome, which results in reduced interactions with serum proteins, and thus reduced opsonization. It has been suggested (Wu *et al.*, 1993) that PEG-PE may make liposomes less likely to be adsorbed on the glycocalyx coat on the plasma membrane of endothelial cells. If transcytosis contributes significantly to the extravasation process, then the PEG coating present on the liposome may, for instance, effectively decrease liposome-cell interaction and limit the rate of passive targeting.

It should be noted that the presence of P388 tumor in the peritoneal cavity had a much greater impact on SA-LUV accumulation in the peritoneal cavity than the strategies of RES blockade and incorporation of PEG-PE. This demonstrates that factors other than circulation lifetime, such as vascular permeability, affect liposome accumulation in the peritoneal cavity. It is known that tumors can secrete a vascular permeability factor that further promotes leakage of circulating macromolecules by increasing intracellular gaps and transendothelial transcytosis processes (Senger *et al.*, 1983; Kohn *et al.*, 1992). The tumor factors which promote blood vessel permeability may prove effective at increasing accumulation of SA-LUV in an extravascular site.

In conclusion, the results presented in this report demon-

strate that liposomes with a surface-associated targeting protein can access sites outside the blood compartment. The circulation lifetime of the carrier affects this extravasation process. However, extravasation is dependent on additional factors that have yet to be identified. Of particular interest is the cell-mediated process that may regulate macromolecule transport across blood vessels. The approaches used here to maximize the circulation lifetime of SA-LUV resulted in increased levels of liposome accumulation in a defined extravascular site, the peritoneal cavity. It should be noted that, in animals bearing an i.p. tumor, almost 7% of the SA-LUV dose could be recovered from the peritoneal cavity 24 hr after i.v. administration. Because studies from this laboratory have focused on developing a liposome targeting approach whereby SA-LUV bind to target cells prelabeled with biotinylated antibodies (Loughrey *et al.*, 1990a, 1993; Longman *et al.*, 1995), we are presently evaluating the ability of extravasated SA-LUV to bind biotin-labeled targets within the peritoneal cavity. We believe that a significant advantage of this two-step procedure is that a single liposomal carrier can be developed for targeting to a variety of pretargeted biotinylated ligands.

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