Pharmaceutical Research, Vol. 10, No. 4, 1993

Research Article

Immunotargeting of Liposomes Containing Lipophilic Antitumor Prodrugs

Atsuhide Mori,[1] Stephen J. Kennel,[2] and Leaf Huang[1,3]

Received August 10, 1992; accepted October 20, 1992

Potential therapeutic applications of recently developed liposomes with a reduced affinity to the reticuloendothelial systems and a prolonged circulation time as targeting systems for lipophilic prodrugs were examined. In these studies, liposomes composed of phosphatidylcholine and cholesterol, additionally containing monosialoganglioside (G_{M1}) or polyethylene glycol conjugated to phosphatidylethanolamine (PEG-PE), were used. Three antitumor lipophilic prodrugs, *N*-trifluoroacetyladriamycin-14-valerate (AD32), araC-diphosphate-diglyceride (araCdPdG), and 3′,5′-*o*-dipalmitoyl-5-fluoro-2′-deoxyuridine (dpFUdR), were used to examine the effect of lipophilic prodrug incorporation into long-circulating liposomes and immunoliposomes on their biodistribution in mouse. Biodistribution studies with antibody-free liposomes containing lipophilic prodrugs showed that the activities of G_{M1} or PEG2000-PE in prolonging the circulation time of liposomes appeared to be preserved in the presence of each of the three lipophilic prodrugs at a drug/lipid molar ratio of 3:97. The effect of lipophilic prodrug incorporation on target binding of immunoliposomes was then examined using a mouse model. Incorporation of AD32 or dpFUdR into immunoliposomes, directed to the normal endothelium, did not affect the targetability of immunoliposomes, suggesting a potential effectiveness of these lipophilic prodrug-containing immunoliposomes in therapy for lung tumors. On the contrary, incorporation of araCdPdG resulted in significantly reduced target binding of immunoliposomes by yet unknown mechanism(s).

KEY WORDS: long-circulating liposome; immunoliposome; lipophilic prodrug; drug delivery.

INTRODUCTION

Efficient cancer chemotherapy requires a high degree of selective localization of cytotoxic drugs in the malignant tissue. In this context, various drug targeting systems have been proposed and investigated extensively for potential therapeutic uses (for a review, see Ref. 1). Liposomes have attracted considerable interest because of their favorable characteristics as drug carriers (for recent reviews, see Ref. 2).

The biodistribution of liposomes *in vivo* has been studied exhaustively in the last decade (for a review, see Ref. 3). These studies have clearly shown that conventional liposomes exhibit a high affinity to the reticuloendothelial system (RES)[4] and thus are inefficient in *in vivo* targeting to cells, tissues, or organs other than the RES. However, this problem has recently been overcome with the development of liposomes with reduced affinities for the RES and prolonged circulation times (for a review, see Ref. 4). Such liposomes are constructed by altering the lipid composition to include a specific amphiphile, such as monosialoganglioside (G_{M1}) or polyethylene glycol conjugated to phosphatidylethanolamine (PEG-PE). Selection of appropriate liposome sizes also can be used for optimization (5). The usefulness of long-circulating liposomes in targeting to non-RES tissues was first demonstrated by Gabizon and Papahadjopoulos (6). Liposomes with modified lipid composition were able to accumulate in solid tumor more efficiently than did conventional liposomes (6).

In addition to the above example of passive targeting of liposomes to non-RES tissues, active targeting of liposomes, which takes advantage of a ligand–receptor interaction, has also been studied extensively. Antibody-directed liposomes, or immunoliposomes, have been exploited due to their high degree of specificity and versatility (for a review, see Ref. 7).

[1] Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261.

[2] Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

[3] To whom correspondence should be addressed at Department of Pharmacology, 13th Floor, Biomedical Science Tower, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261.

[4] Abbreviations used: AD32, *N*-trifluoroacetyladriamycin-14-valerate; araCdPdG, araC-diphosphate-diglyceride; biotin-cap-PE, biotinamidocaproyl-phosphatidylethanolamine; Chol, cholesterol; dpFUdR, 3′,5′-*o*-dipalmitoyl-5-fluoro-2′-deoxyuridine; DTPA-SA, diethylenetriamine pentaacetic acid distearylamide complex; G_{M1}, monosialoganglioside; NGPE, *N*-glutaryl phosphatidylethanolamine; PBS, phosphate-buffered saline; PC, egg phosphatidylcholine; PEG, polyethylene glycol; PEG-PE, dioleoyl *N*-(monomethoxy polyethylene glycol succinyl) phosphatidylethanolamine; RES, reticuloendothelial system.

0724-8741/93/0400-0507$07.00/0

We have been using the mouse lung endothelium model for characterization of *in vivo* targeting of immunoliposomes. Monoclonal antibody 34A (MoAb 34A) binds specifically with a glycoprotein antigen, thrombomodulin, expressed at high concentrations on the lumenal surface of the capillary endothelial cells of mouse lung (8). The i.v. injected MoAb 34A-bearing liposomes (34A-liposomes) gain direct access to the lung target and accumulate primarily in the lung and secondarily in the liver (9). We have also shown that 34A-liposomes designed to exhibit a reduced affinity to the RES by inclusion of G_{M1} (10,11) or PEG2000-PE (12) in the lipid composition exhibit more efficient target binding and also longer retention at the lung target than immunoliposomes with conventional lipid compositions. Thus, the use of liposomes with prolonged circulation seems to result in improved targeting to non-RES tissues.

In the present study, we have examined the drug delivery potential of the above novel classes of liposomes and immunoliposomes using three lipophilic derivatives of cytotoxic drugs, i.e., *N*-trifluoroacetyladriamycin-14-valerate (AD32) (13), araC-diphosphate-diglyceride (araCdPdG) (14), and 3′,5′-*o*-dipalmitoyl-5-fluoro-2′-deoxyuridine (dpFUdR) (15). Lipophilic prodrugs were chosen in this study because of their excellent incorporation efficiency into liposomes. However, it is possible that incorporation of prodrugs into liposome membranes would modify the surface characteristics of the liposomes. It is shown here that prodrugs can be delivered to lung endothelium efficiently by liposomes with modified lipid compositions.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Cholesterol (Chol), polyethylene glycol (PEG) (average MW 8000), and dextran (average MW 515,000) were from Sigma Chemical Co. (St. Louis, MO). Bovine monosialoganglioside (G_{M1}) was obtained from Matreya Inc. (Mount Pleasant, PA), and PEG (MW 2,000) was from Nippon Oil & Fats Co., Ltd. (Tsukuba, Japan). *N*-trifluoroacetyladriamycin-14-valerate (AD32) and araC-diphosphate-diglyceride (araCdPdG) were kindly provided by Dr. M. Israel and Dr. J. G. Turcotte, respectively, and 3′,5′-*o*-dipalmitoyl-5-fluoro-2′-deoxyuridine (dpFUdR) was from Dr. M. van Borssum Waalkes and Dr. G. L. Scherphof. Synthesis of dioleoyl *N*-(monomethoxypolyethyleneglycol succinyl)phosphatidylethanolamine (PEG-PE) (16), diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) (17), *N*-glutaryl-phosphatidylethanolamine (NGPE) (11), and biotinamidocaproyl-phosphatidylethanolamine (biotin-cap-PE) (18) has been described previously. Radiolabeling of DTPA-SA with ^{111}In was performed as described previously (16). Rat MoAb 34A was purified from nu/nu mouse ascites fluid (8) and was radiolabeled with ^{125}I using the Iodo-Gen method (Rockford, IL) and conjugated with NGPE as described previously (19).

Liposome Preparations

Large unilamellar liposomes composed of PC/Chol (10:5, mol/mol), PC/Chol/G_{M1} (10:5:1, mol/mol), and PC/Chol/PEG2000-PE (10:5:1, mol/mol) with or without a lipophilic prodrug (AD32, araCdPdG or dpFUdR) at 3.0 mol% of the lipid mixture were prepared by the extrusion method. Briefly, the solvent free lipid mixture containing ^{111}In-labeled DTPA-SA, as a nonexchangeable and nonmetabolizable lipid marker (17), at 1.0 mol% of the lipid mixture was hydrated with phosphate-buffered saline (PBS) (pH 7.5) overnight. The liposome suspension (2 mg of lipid/ml of PBS) was extruded, at room temperature, 8–10 times through stacked Nucleopore membranes (0.4- and 0.2-μm pore size) using a syringe-type filter holder (Whatman, OL) to generate liposomes with homogeneous size distributions.

The MoAb 34A-bearing liposomes (34A-liposomes) with the modified lipid compositions were prepared by the detergent-dialysis method as described previously (19). Briefly, the lipid mixture containing 1.0 mol% ^{111}In-labeled DTPA-SA, solubilized with octylglucoside in MES buffer (pH 5.0) (lipid/octylglucoside = 1:5, mol/mol), was mixed with MoAb 34A conjugated with NGPE (normally, antibody/lipid = 1:4, w/w), and detergent was removed by dialysis for 36 hr at 4°C. The resulting 34A-liposomes were extruded as described above, and the unincorporated antibody was then removed by column chromatography on BioGel A1.5M (Bio-Rad Laboratories, NY). The liposome fractions were pooled and diluted to 1 mg of lipid/mL of PBS. The antibody/lipid weight ratio of the immunoliposomes was determined from calculations with known specific activities of ^{111}In-lipids and ^{125}I-antibody. The liposome suspension was further diluted to ~20 μg of lipid/mL of PBS, and liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, FL) and is expressed as an average diameter with SD.

Liposome Partitioning Assay

An aqueous two-phase system composed of PEG and dextran (20) was used in this study. Briefly, both PEG8000 and dextran were dissolved at 5% (w/w) in 0.01 *M* Na-phosphate buffer (pH 6.8) containing 0.15 *M* NaCl ("non-charged" phase system), and the phase system was allowed to equilibrate overnight at room temperature. The PEG-rich upper and dextran-rich lower phases were then separated and kept at 4°C. For the liposome partitioning assay, ^{111}In-labeled liposomes (0.1 mg lipid in 50 μl PBS, pH 7.5) were mixed with equal volumes (1 mL) of the upper and lower phases in a 50 × 10-mm tube. Immediately after mixing by repeated inversion for 1 min, 50 μL of the mixture was sampled for total radioactivity counting. The mixture was left at room temperature for 30 min to allow for phase separation, and 100 μL of each phase was sampled to determine liposome partitioning. The amount of liposomes localized at horizontal interface was obtained by subtracting the sum of the radioactivity in the upper and lower phases from the total radioactivity. Data are expressed as the percentage of total liposomes in the upper and lower phases and interface.

Liposome Agglutination Assay

Liposomes used in this study were prepared to include biotin-cap-PE at 2.5 mol% of the lipid mixture (21). Agglutination was initiated by mixing liposomes (60 μg phospholipid in 560 μL PBS, pH 7.5) with streptavidin (10 μg) in a

microcuvette. The increase in turbidity was measured by optical density at 440 nm after 12 min of incubation. Data are expressed as the relative turbidity of the test liposomes to that of control liposomes.

Liposome Biodistribution Study

^{111}In-Labeled liposomes with various lipid compositions were injected i.v. into male Balb/c mice (6–8 weeks old) at a dose of 0.2–0.4 mg lipid per mouse in 0.2 mL of PBS. Administration of liposomes in this dose range does not cause saturation of the liposome uptake by the RES, and the biodistribution of liposomes is independent of both the amount and the number of injected liposomes. At specified time intervals, mice were anesthetized, bled by retroorbital puncture, and then sacrificed by cervical dislocation and dissected. Blood and major organs including spleen, liver, lung, heart, and kidney were collected and weighed. Biodistribution of liposomes was determined by analyses of ^{111}In radioactivity in each organ using a Beckman gamma-counter. Data are expressed as the percentage of the total injected dose of liposomes in each organ. Accumulation of liposomes in the RES is expressed as the sum of accumulation in the liver and the spleen. Liposome levels in the blood were determined by assuming that the blood volume of the mouse is 7.3% of the total body weight (22).

RESULTS

Incorporation of Lipophilic Prodrugs into Liposomes

Incorporation of all three lipophilic prodrugs at 3.0 mol% of the lipid mixture did not cause aggregation or precipitation of drugs and/or liposomes before or after the extrusion procedure, indicating complete incorporation of lipophilic prodrugs at this concentration. Incorporation of lipophilic prodrugs at higher concentrations inhibited the extrusion procedure due to insoluble unincorporated drug molecules. To examine the long-term stability of liposomes containing lipophilic prodrugs, liposomes were stored at 4°C and the diameters of liposomes were periodically determined by dynamic laser light scattering. Liposome size (152–227 nm in average diameters), irrespective of lipid compositions and lipophilic prodrug incorporation, tends to increase only slightly (up to 15% of the original diameter) after 4 weeks of storage, indicating that lipophilic prodrugs do not significantly affect liposome stability *in vitro*.

Effect of Lipophilic Prodrug Incorporation on Surface Polarity of Liposomes

One of the important factors determining the *in vivo* distribution of liposomes is their surface polarity (20). We have thus examined the effect of lipophilic prodrug incorporation on liposome surface polarity using an aqueous two-phase partitioning assay. This system (PEG/dextran) with a selected conditions can be used to monitor either surface charge or hydrophobicity of liposomes (for a review, see Ref. 23). To examine the relative surface hydrophobicity of liposomes containing lipophilic prodrugs, a "noncharged" phase system was used. The system is insensitive to the surface charge of the liposomes, and partitioning behavior of liposomes depends strongly on the surface hydrophobicity of liposomes (23). Three liposomal formulations, PC/Chol, PC/Chol/G_{M1}, and PC/Chol/PEG2000-PE, with or without lipophilic prodrugs at 3.0 mol% were prepared by the extrusion method, and average diameters of these liposomes ranged from 152 to 227 nm. Partitioning of liposomes was examined 30 min after mixing, and data are expressed as percentage of total liposomes in two phases and at interface, together with a respective upper/lower ratio (Table I). Liposomes composed of PC and Chol are found mostly at the interface (54%) and in the lower phase (42%). Inclusion of G_{M1} into PC/Chol liposomes did not alter this partitioning behavior. On the contrary, inclusion of PEG2000-PE resulted in enhanced partitioning of the liposomes into the upper phase (81%), indicating an enhanced hydrophilicity of

Table I. Partitioning of Liposomes Containing Lipophilic Prodrugs in the Aqueous Two-Phase System[a]

Lipid composition[b]	Drug incorporated[c]	% liposome added[d]			Upper/lower	Lower/interface
		Upper phase	Interface	Lower phase		
PC/Chol	—	3.9 (0.1)	54.4 (1.0)	41.7 (0.9)	0.09	0.77
PC/Chol	AD32	4.1 (0.2)	43.5 (2.5)	52.5 (2.5)	0.08	1.21
PC/Chol	AraCdPdG	3.4 (0.2)	34.2 (1.3)	62.4 (1.5)	0.05	1.83
PC/Chol	dpFUdR	5.8 (0.5)	63.7 (3.0)	30.5 (2.6)	0.19	0.48
PC/Chol/GM1	—	3.2 (0.1)	58.8 (3.1)	38.0 (3.0)	0.08	0.65
PC/Chol/GM1	AD32	3.6 (0.5)	56.6 (3.0)	39.8 (2.5)	0.09	0.70
PC/Chol/GM1	AraCdPdG	3.4 (0.0)	42.7 (1.4)	53.9 (1.4)	0.06	1.26
PC/Chol/GM1	dpFUdR	3.6 (0.1)	62.9 (3.7)	33.6 (3.6)	0.11	0.53
PC/Chol/PEG2000-PE	—	81.4 (2.4)	14.0 (2.6)	4.6 (0.2)	17.7	0.33
PC/Chol/PEG2000-PE	AD32	80.0 (0.8)	14.9 (0.8)	5.1 (0.6)	15.7	0.34
PC/Chol/PEG2000-PE	AraCdPdG	82.0 (3.6)	13.9 (3.8)	4.1 (0.2)	20.0	0.30
PC/Chol/PEG2000-PE	dpFUdR	78.9 (1.4)	17.0 (1.3)	4.1 (0.3)	19.2	0.24

[a] The phase system composed of 5% (w/w) PEG/5% (w/w) dextran in 0.01 *M* Na-phosphate buffer (pH 6.8) containing 0.15 *M* NaCl was used.

[b] Molar ratios are as described under Materials and Methods.

[c] Lipophilic prodrugs were incorporated at 3.0 mol% of the lipid mixture.

[d] Data are expressed as mean (SD); $n = 3$.

PEG2000-PE-containing liposomes. Partitioning of PC/Chol and PC/Chol/G_{M1} liposomes into the upper phase did not change upon incorporation of each of the three lipophilic prodrugs. However, incorporation of araCdPdG affected partitioning of liposomes between the interface and the lower phase. Incorporation of araCdPdG into PC/Chol and PC/Chol/G_{M1} liposomes resulted in reduced localization at interface with a concomitant increase in partitioning into the lower phase. Incorporation of each of the three lipophilic prodrugs into PC/Chol/PEG2000-PE liposomes did not affect the partitioning of the parent liposomes.

Biodistribution of Antibody-Free Liposomes Containing Lipophilic Prodrugs

We then examined whether prodrug incorporation into liposomes affects the activities of G_{M1} and PEG2000-PE in prolonging the circulation time of liposomes. In this study, three liposomal formulations with or without 3.0 mol% each lipophilic prodrug were prepared, with the average diameter ranging from 161 to 231 nm. Liposomes were injected i.v. into mice, and the biodistribution was examined 3 hr later. This time point was chosen because liposomes of this size range exhibit blood clearance half-lives between 0.5 and 7 hr (12). Data are expressed as the percentage of injected dose of liposomes in the blood and the RES, together with the respective RES/blood ratios (Table II). Conventional liposomes, composed of PC/Chol, cleared rapidly from the circulation and accumulated preferentially in the RES, with a RES/blood ratio of 5.3 at 3 hr post injection. Inclusion of G_{M1} or PEG2000-PE in the lipid composition resulted in significantly increased level of liposomes in the blood, with concomitant decreased accumulation in the RES. The RES/blood ratios of G_{M1}- and PEG2000-PE-containing liposomes were 0.52 and 0.58, respectively, indicating a similar activity of G_{M1} and PEG2000-PE in prolonging the circulation time of liposomes. Inclusion of AD32 or dpFUdR in PC/Chol liposomes did not cause a measurable alteration in their biodistribution behavior. On the other hand, inclusion of araCdPdG in PC/Chol liposomes increased the liposome level in the circulation significantly, with a RES/blood ratio of 1.62. Data in Table II also show that incorporation of each of the three lipophilic prodrugs into G_{M1}- or PEG2000-PE-containing liposomes did not significantly affect the amount of the parent liposomes remaining in the circulation, indicating that the activities of G_{M1} and PEG2000-PE in prolonging the circulation time of liposomes were preserved even in the presence of the lipophilic prodrugs in the liposomes.

Incorporation of Amphipathic Antibody into Lipophilic Prodrug-Containing Liposomes

During the dialysis, extrusion, and chromatographic purification steps in the immunoliposome preparation, recovery of lipids was 61–91%, as determined with ^{111}In-labeled DTPA-SA (Table III). Nevertheless, the calculated relative incorporation ratio of amphipathic antibody molecules into liposomes after normalizing for the loss of lipids was nearly the same (34–38%) in all liposome formulations. Thus, the inclusion of lipophilic prodrug does not affect the incorporation of amphipathic antibody molecules into liposomes.

Target Binding of Immunoliposomes Containing Lipophilic Prodrugs

Effect of lipophilic prodrug incorporation into immunoliposomes on their target binding was examined using the MoAb 34A system. In this study, 34A-liposomes with the lipid compositions of PC/Chol/G_{M1} and PC/Chol/PEG2000-PE with or without 3.0 mol% lipophilic prodrug were prepared and injected i.v. into mice. The antibody/lipid ratios and average diameters of 34A-liposomes used in this study ranged from 1:11.3 to 1:13.9 (w/w) and from 137 to 178 nm, respectively. Lung binding and the RES uptake of 34A-liposomes were examined 2 hr after injection, and values are

Table II. Biodistribution of Liposomes Containing Lipophilic Prodrugs[a]

Lipid composition[b]	Drug incorporated[c]	Average diameter (nm)	% injected dose[d]			RES/blood ratio
			Blood	RES	Other[e]	
PC/Chol	—	208 (64)	13.8 (4.4)	73.0 (6.2)	2.2 (0.6)	5.30
PC/Chol	AD32	194 (71)	14.4 (0.7)	69.7 (3.2)	1.6 (0.2)	4.84
PC/Chol	AraCdPdG	231 (86)	32.3 (4.0)	52.2 (4.1)	3.6 (0.2)	1.62
PC/Chol	dpFUdR	198 (66)	22.0 (4.4)	64.8 (5.8)	2.8 (0.2)	2.95
PC/Chol/GM1	—	205 (65)	54.1 (0.6)	28.2 (1.6)	4.9 (0.7)	0.52
PC/Chol/GM1	AD32	184 (61)	54.3 (1.9)	28.5 (1.9)	5.3 (0.4)	0.52
PC/Chol/GM1	AraCdPdG	187 (57)	45.6 (0.9)	39.1 (1.9)	3.5 (0.2)	0.86
PC/Chol/GM1	dpFUdR	204 (71)	50.1 (3.2)	34.5 (1.9)	4.3 (0.6)	0.69
PC/Chol/PEG2000-PE	—	184 (61)	50.9 (0.9)	29.7 (0.7)	4.4 (0.8)	0.58
PC/Chol/PEG2000-PE	AD32	161 (52)	52.3 (3.8)	27.1 (3.1)	4.4 (0.5)	0.52
PC/Chol/PEG2000-PE	AraCdPdG	160 (50)	47.4 (3.0)	30.3 (1.0)	4.3 (1.0)	0.64
PC/Chol/PEG2000-PE	dpFUdR	178 (59)	48.3 (4.3)	31.2 (2.1)	4.0 (0.4)	0.65

[a] In-Labeled liposomes with the indicated lipid composition and average diameter were injected i.v. into mice at a dose of 0.4 mg lipid/mouse. Biodistribution was examined 3 hr after injection.
[b] Molar ratios are as described under Materials and Methods.
[c] Lipophilic prodrugs were incorporated at 3.0 mol% of the lipid mixture.
[d] Data are expressed as mean (SD), $n = 3$.
[e] Others include lung, heart, and kidney.

Table III. Incorporation of Amphipathic Antibody into Liposomes Containing Lipophilic Prodrug[a]

Lipid composition[b]	Drug incorporated[c]	Recovery (%)[d]		Relative incorporation of 34A into liposomes (%)[e]
		34A	Lipids	
PC/Chol/GM1	—	31.2	90.6	34.4
PC/Chol/GM1	AD32	21.6	61.2	35.3
PC/Chol/GM1	AraCdPdG	33.7	89.6	37.6
PC/Chol/GM1	dpFUdR	28.3	79.3	35.7
PC/Chol/PEG2000-PE	—	29.0	76.9	37.7
PC/Chol/PEG2000-PE	AD32	25.3	72.9	34.7
PC/Chol/PEG2000-PE	AraCdPdG	27.1	70.6	38.4
PC/Chol/PEG2000-PE	dpFUdR	26.6	72.4	36.7

[a] 34A-Liposomes with the indicated lipid composition were prepared by the detergent-dialysis method as described under Materials and Methods.
[b] Molar ratios are as described under Materials and Methods.
[c] Lipophilic prodrugs were incorporated at 3.0 mol% of the lipid mixture.
[d] Recoveries of 34A and lipids were determined by radioactivity counting of ^{125}I and ^{111}In, respectively.
[e] Relative incorporation values of 34A into liposomes after normalizing for the recovery of lipids.

expressed as the percentage of injected dose (Table IV). The target binding of the control 34A-liposomes without lipophilic prodrugs showed 48.6 and 49.3% for G_{M1}- and PEG2000-PE-containing 34A-liposomes, respectively. Inclusion of AD32 or dpFUdR in these two immunoliposome formulations did not cause a significant change in lung binding. However, incorporation of araCdPdG resulted in a significantly decreased lung binding (25.5%), with a concomitant increase in the RES uptake of 34A-liposomes when this prodrug was incorporated into PC/Chol/G_{M1} liposomes. On the other hand, incorporation of araCdPdG into PC/Chol/PEG2000-PE liposomes lowered the accumulation of liposomes in the lung only slightly.

To verify the interference of araCdPdG on target binding of G_{M1}-containing 34A-liposomes, 34A-liposomes with lipid compositions of PC/Chol or PC/Chol/G_{M1} with or without 3.0 mol% araCdPdG were prepared with relatively low antibody densities (antibody/lipid, 1:29–35, w/w) and were injected i.v. into mice. Lung binding, RES uptake, and blood level of 34A-liposomes were examined at different times after injections (Fig. 1). Clearly, lung binding of araCdPdG-containing 34A-liposomes was lower than that of the respective control 34A-liposomes without araCdPdG. The liver uptake of 34A-liposomes also increased when araCdPdG was included in the lipid composition, although blood clearance profiles were identical between the control and the araCdPdG-containing 34A-liposomes in both liposome formulations.

One of the potential mechanisms of araCdPdG in lessening target binding of 34A-liposomes is that this prodrug presents a steric barrier on the liposome surfaces such that the antibody/antigen interactions are hindered. To test this possibility, a liposome agglutination assay was used. Liposomes used in this study were prepared to include biotin-cap-PE at 2.5 mol% of the lipid mixture. When a steric barrier is produced by an additional lipid component on liposome surface, it reduces an effective interaction of streptavidin with liposomal biotin-cap-PE, resulting in a reduced agglutination (12,21). Thus, the relative steric barrier activity of a lipophilic prodrug can be assessed using this assay. Results showed that araCdPdG, as well as AD32 and dpFUdR, did not affect liposome agglutination (Table V),

Table IV. Target Binding of 34A-Liposomes Containing Lipophilic Prodrugs[a]

Lipid composition[b]	Drug incorporated[c]	34A:lipid (w/w)	Average diameter (nm)	% injected dose[d]		
				Lung	RES	Blood
PC/Chol/GM1	—	1:13.9	137 (40)	48.6 (1.3)	31.6 (1.2)	2.7 (0.4)
PC/Chol/GM1	AD32	1:12.3	139 (40)	40.8 (2.0)	47.3 (2.2)	1.5 (0.3)
PC/Chol/GM1	AraCdPdG	1:13.6	146 (47)	25.5 (1.3)	58.2 (7.9)	2.1 (0.5)
PC/Chol/GM1	dpFUdR	1:13.8	160 (51)	42.3 (1.9)	44.2 (1.6)	2.2 (0.1)
PC/Chol/PEG2000-PE	—	1:11.3	158 (50)	49.3 (1.3)	25.7 (1.7)	5.6 (0.7)
PC/Chol/PEG2000-PE	AD32	1:12.4	146 (46)	47.9 (0.8)	27.9 (0.8)	7.0 (0.3)
PC/Chol/PEG2000-PE	AraCdPdG	1:11.7	153 (53)	41.5 (1.7)	33.3 (0.4)	5.8 (1.1)
PC/Chol/PEG2000-PE	dpFUdR	1:12.1	178 (57)	45.6 (2.4)	29.0 (1.8)	6.1 (1.0)

[a] In-Labeled 34A-liposomes with the indicated lipid composition, 34A/lipid ratio, and average diameter were injected i.v. into mice at a dose of 0.3 mg lipid/mouse. Biodistribution was examined 2 hr after injections.
[b] Molar ratios are as described under Materials and Methods.
[c] Lipophilic prodrugs were incorporated at 3.0 mol% of the lipid mixture.
[d] Data are expressed as mean (SD), $n = 3$.

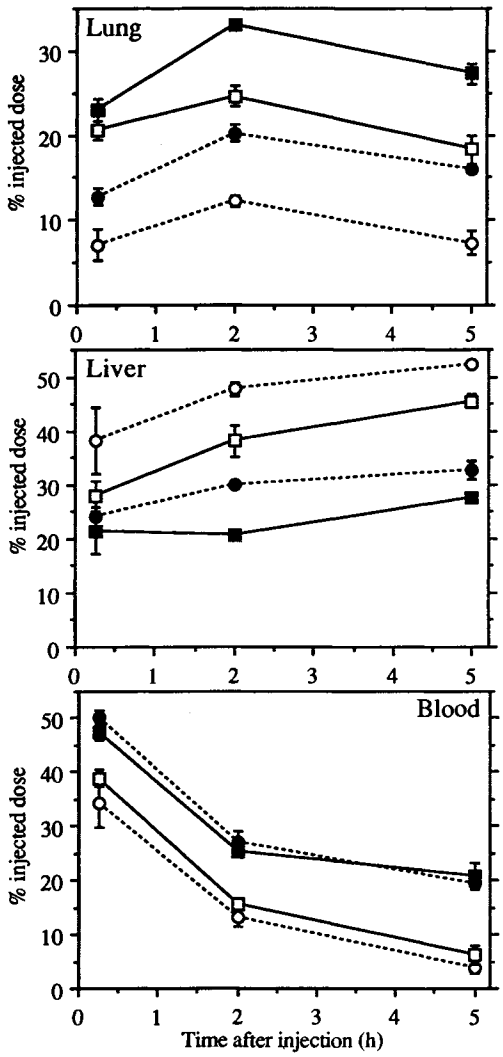

Fig. 1. Lung binding and the RES uptake of 34A-liposomes containing araCdPdG. ^{111}In-Labeled 34A-liposomes were injected i.v. into mice. Liposome levels in the lung, RES, and blood were examined at indicated intervals after injections and are expressed as percentage of injected dose. Bars represent SD (n = 3). Lipid composition, antibody-to-lipid ratio (w/w), and average diameter of 34A-liposomes were as follows: (□) PC/Chol (10:5, mol/mol), 1:33, 158 nm; (○) PC/Chol/araCdPdG (10:5:0.46, mol/mol), 1:29, 157 nm; (■) PC/Chol/G_{M1} (10:5:1, mol/mol, 1:31, 154 nm; (●) PC/Chol/G_{M1}/araCdPdG (10:5:1:0.5, mol/mol), 1:35, 154 nm, respectively.

indicating only weak steric barrier activities of all three lipophilic prodrugs.

DISCUSSION

An advantage in using lipophilic drugs, instead of water-soluble drugs, in liposomal targeting systems is their high degrees of incorporation into liposomes due to their hydrophobic property. While water-soluble drugs generally show poor incorporation efficiency, virtually complete incorporation of lipophilic prodrugs can be achieved by selecting a drug concentration not exceeding the saturation level in the lipid bilayers. From the pharmaceutical viewpoint, this would be especially advantageous since no additional purification step is necessary to separate liposomal drug formulations from unincorporated drug molecules. The use of liposomal systems not only provides a way of drug solubilization for i.v. administration but also may result in altered biodistribution of lipophilic prodrugs, depending on the liposomal system to be used. Since a large portion of lipophilic prodrugs is incorporated into the bilayer phase of liposomes, it is likely that some surface properties of liposomes and thus their biodistribution profiles could be altered. We have previously reported that G_{M1} and PEG2000-PE show relatively weak steric barrier activities on liposome surfaces and thus do not interfere with target binding of immunoliposomes (12). Furthermore, immunoliposomes containing these amphiphiles showed enhanced target binding due to their activities in prolonging the circulation time of liposomes (10–12). In the present study, we have examined whether incorporation of lipophilic prodrugs into liposomes affects (i) *in vitro* stability of liposomes, (ii) surface hydrophobicity of liposomes, (iii) activities of G_{M1} and PEG2000-PE in prolonging the circulation time of liposomes, (iv) preparation of immunoliposomes, and (v) target binding of immunoliposomes. These studies are the first step toward potential uses of G_{M1}- or PEG2000-PE-containing liposomes and immunoliposomes as a delivery system for lipophilic prodrugs.

Table V. Effect of Lipophilic Prodrug Incorporation on Streptavidin-Induced Agglutination of Liposomes Containing Biotin-Cap-PE

Lipid composition[a]	Drug incorporated[b]	Relative turbidity[c]
PC/Chol/GM1	—	1.00
PC/Chol/GM1	AD32	0.92
PC/Chol/GM1	AraCdPdG	0.94
PC/Chol/GM1	dpFUdR	1.01

[a] Molar ratios are as described under Materials and Methods.
[b] Lipophilic prodrugs were incorporated at 3.0 mol% of the lipid mixture.
[c] Data are expressed as relative turbidity of the test liposomes to that of control liposomes (PC/Chol/GM1).

Data in Table II clearly indicated that incorporation of lipophilic prodrugs in liposomes at 3.0 mol% of the lipid mixture does not affect the activities of G_{M1} and PEG2000-PE in prolonging the circulation time of liposomes. Since liposomes with prolonged circulation times accumulate more effectively in solid tumors than do conventional liposomes (6), these liposomes containing lipophilic prodrugs should also accumulate effectively in the tumor. We have tested the ability of lipophilic prodrug-containing, long-circulating liposomes to accumulate in a mouse solid tumor model (EMT-6). Results showed that, regardless of the presence/absence of lipophilic prodrugs, long-circulating liposomes containing G_{M1} or PEG5000-PE accumulated in the tumor more efficiently than did the conventional liposomes without G_{M1} or PEG5000-PE (Mori and Huang, unpublished data). Recently, other investigators have reported improved therapeutic indices of cytotoxic drugs using long-circulating liposomes as compared to conventional liposomes as drug carriers (24,25).

Although mechanisms of G_{M1} or PEG-PE in prolonging the circulation time of liposomes are not clearly understood,

increased hydrophilicity and/or steric barriers produced on the liposome surface by these amphiphiles seems to be involved in liposome stabilization event in the blood (for a review, see Ref. 4), although we have previously suggested the different mechanisms involved in G_{M1} and PEG-PE actions (12). Senior *et al.* (20) have suggested that PEG covalently attached to liposomes exerts its activity in prolonging the circulation time of liposomes by increasing the surface hydrophilicity of liposomes. Our liposome partitioning assay also showed that PEG2000-PE-containing liposomes exhibit enhanced hydrophilicity, as they partition predominantly into the upper PEG phase (Table I). However, inclusion of G_{M1}, another amphiphile having an activity in prolonging the circulation time of liposomes, into PC/Chol liposomes does not affect surface polarity of liposomes (Table I), suggesting that altered surface hydrophobicity may not be involved in the mechanism of action for G_{M1}. Tagesson *et al.* (26) have reported previously that liposomes with a high affinity to the dextran phase in the noncharged phase system were cleared from the circulation more rapidly. However, our biodistribution study of araCdPdG-containing liposomes showed that this prodrug also shows some activity in prolonging the circulation time of liposomes, despite the higher affinity of these liposomes to the dextran phase than the control liposomes (Table I). Although the relative contribution of liposome surface hydrophilicity to the mechanism of prolonged circulation is not clear, the above results indicate that it is not the only determining factor for long-circulating liposomes.

Data in Table IV indicate that at least two lipophilic prodrugs, AD32 and dpFUdR, can be incorporated into immunoliposomes without lessening their target binding efficiency. It should be noted that, since target binding efficiency of 34A-liposomes is a function of both the lipid composition of liposomes and the antibody/lipid ratio (9–12), actual target binding can be further increased by using a higher antibody density on the liposomes. Thus, a majority of administered lipophilic prodrugs could be delivered to the lung target via 34A-liposomes. This strategy seems to be effective especially for drugs with narrow therapeutic indices. Liposomal formulations of dpFUdR have been studied by several investigators (15,27,28). van Borssum Waalkes and Scherphof (28) have reported that, although an antitumor activity of liposomal dpFUdR is greatly enhanced in liposomal formulations compared to its parent drug, FUdR, liposomal dpFUdR also exhibits an increased toxicity to the host due to the predominant localization of the drug in the liver. Since 34A-liposomes are designed to deliver drugs to the lung, while minimizing drug accumulation in the liver, the combination of 34A-liposomes with dpFUdR seems to be a potentially effective approach in therapy for lung tumors. In the concept of this organ-specific immunoliposomes (29), the lipophilic prodrugs are expected to be released/transferred from the bound immunoliposomes (i.e., via the lipid exchange mechanism between the liposomes and the cell membranes), diffuse through cell membranes, and reach the tumor cells in the same organ where the prodrug can be converted to the parent drug. We have been studying the therapeutic potential of dpFUdR-containing 34A-liposomes using an experimental mouse pulmonary metastasis model. Preliminary results showed the effectiveness of these immunoliposomes in prolonging the survival time of mice compared to the free drug (29).

Incorporation of araCdPdG into immunoliposomes caused reduced target binding (Table IV and Fig. 1). The mechanism of araCdPdG in lessening the target binding of immunoliposomes is not known. We have previously observed similar effects on target binding of immunoliposomes by phosphatidylserine (PS) and PEG5000-PE. PS is well-known to exhibit so-called "opsonin-like activity": its incorporation into liposomes makes them much more susceptible to uptake by the RES (30). PS-containing immunoliposomes are rapidly cleared from the circulation, thus resulting in a reduced target binding (10). On the other hand, PEG5000-PE exerts its inhibition effect on target binding of immunoliposomes via its overly strong steric barrier activity on the liposome surfaces, which presumably interferes with interactions between the antibody and the target antigen (12,21). However, it is unlikely that araCdPdG reduces target binding of immunoliposomes by the same mechanisms, since (i) araCdPdG does not affect the activity of G_{M1} in prolonging the circulation time of liposomes (Table II), and (ii) a streptavidin-induced agglutination assay of liposomes containing biotin-cap-PE showed that liposome agglutination was not inhibited by the incorporation of araCdPdG, indicating that the steric barrier activity of araCdPdG was negligible (Table V). It is possible that the presence of araCdPdG on the liposome surfaces somehow alters the orientation of amphipathic antibody molecules such that the Fc region of the antibody is more exposed on the liposome surfaces and the antigen binding site is less available for target binding. It is also possible that the free amino group of the ara-C moiety may interact with lipid components and/or antibody molecules on the liposome surfaces, resulting in phase separation and thus reduced target binding efficiency of immunoliposomes. In this regard, it is of interest to test the lipophilic prodrug of ara-C with a blocked amino group (e.g., N^4-acyl derivatives) for immunotargeting of liposomes. Alternatively, liposomes containing araCdPdG are less hydrophilic (Table I) and might attract an increased amount of serum proteins to coat liposome surfaces which then in turn modify the antibody orientation. More experiments are needed to test these hypotheses.

ACKNOWLEDGMENTS

We thank Dr. M. Israel and Dr. J. G. Turcotte for providing AD32 and araCdPdG, respectively and Dr. M. van Borssum Waalkes and Dr. G. L Scherphof for dpFUdR. We also wish to thank Dr. A. L. Klibanov for helpful discussion. This work was supported by NIH Grants CA 24553 and AI 29893.

REFERENCES

1. P. K. Gupta. Drug targeting in cancer chemotherapy: A clinical perspective. *J. Pharm. Sci.* 79:949–962 (1990).
2. M. J. Ostro. *Liposomes: From Biophysics to Therapeutics*, Marcel Dekker, New York, 1987.
3. G. L. Scherphof. In vivo behavior of liposomes: Interactions with the mononuclear phargocyte system and implications for drug targeting. In R. L. Juliano (ed.), *Targeted Drug Delivery*, Springer-Verlag, New York, 1991, pp. 285–327.

4. A. L. Klibanov and L. Huang. Long-circulating liposomes: Development and perspectives. *J. Liposome Res.* 2:321–334 (1992).
5. D. Liu, A. Mori, and L. Huang. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of G_{M1}-containing liposomes. *Biochem. Biophys. Acta* **1104**:95–101 (1992).
6. A. Gabizon and D. Papahadjopoulos. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA* 85:6949–6953 (1988).
7. S. Wright and L. Huang. Antibody-directed liposomes as drug-delivery vehicles. *Adv. Drug Deliv. Rev.* 3:343–389 (1989).
8. V. A. Ford, C. Stringer, and S. J. Kennel. Thrombomodulin is preferentially expressed in Balb/c lung microvessels. *J. Biol. Chem.* 267:5446–5450 (1992).
9. K. Maruyama, E. Holmberg, S. J. Kennel, A. Klibanov, V. P. Torchilin, and L. Huang. Characterization of in vivo immunoliposome targeting to pulmonary endothelium. *J. Pharm. Sci.* 79:978–984 (1990).
10. K. Maruyama, S. J. Kennel, and L. Huang. Lipid composition is important for highly efficient target binding and retention of immunoliposomes. *Proc. Natl. Acad. Sci. USA* 87:5744–5748 (1990).
11. D. C. Litzinger and L. Huang. Biodistribution and immunotargetability of ganglioside-stabilized diolelylphosphatisylethanolamine liposomes. *Biochem. Biophys. Acta* **1104**:179–187 (1992).
12. A. Mori, A. L. Klibanov, V. P. Torchilin, and L. Huang. Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside G_{M1} on the circulation time of liposomes and on the target binding of immunoliposomes *in vivo*. *FEBS Lett.* **284**:263–266 (1991).
13. M. Israel, E. J. Modest, and E. Frei, III. N-Trifluoroacetyladriamycin-14-valerate, an analog with greater experimental antitumor activity and less toxicity than adriamycin. *Cancer Res.* 35:1365–1368 (1975).
14. J. G. Turcotte, S. P. Srivastava, J. M. Steim, P. Calabresi, L. M. Tibbetts, and M. Y. Chu. Cytotoxic liponucleotide analogs. II. Antitumor activity of CDP-diacylglycerol analogs containing the cytosine arabinoside moiety. *Biochim. Biophys. Acta* **619**:619–631 (1980).
15. R. A. Schwendener, A. Supersaxo, W. Rubas, H. G. Weder, H. R. Hartmann, H. Schott, A. Ziegler, and H. Hengartner. 5′-*o*-Parmitoyl- and 3′,5′-*o*-dipalmitoyl-5-fluoro-2′-deoxyuridine—novel lipophilic analogues of 5′-fluoro-2′-deoxyuridine: Synthesis, incorporation into liposomes and preliminary biological results. *Biochem. Biophys. Res. Commun.* **126**:660–666 (1985).
16. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* **268**:235–237 (1990).
17. G. Kabalka, E. Buonocore, K. Hubner, T. Moss, N. Norley, and L. Huang. Gadolinium-labeled liposomes: Targeted MR contrast agents for the liver and spleen. *Radiology* **163**:255–258 (1987).
18. A. L. Klibanov, A. A. Bogdanov, Jr., V. P. Torchilin, and L. Huang. Biotin-bearing pH-sensitive liposomes: High-affinity binding to avidin layer. *J. Liposome Res.* **1**:233–244 (1989).
19. E. Holmberg, K. Maruyama, D. C. Litzinger, S. Wright, M. Davis, G. W. Kabalka, S. J. Kennel, and L. Huang. Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. *Biochem. Biophys. Res. Commun.* **165**:1272–1278 (1989).
20. J. Senior, C. Delgado, D. Fisher, C. Tilcock, and G. Gregoriadis. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: Studies with poly(ethylene glycol)-coated vesicles. *Biochim. Biophys. Acta* **1062**:77–82 (1991).
21. A. L. Klibanov, K. Maruyama, A. M. Beckerleg, V. P. Torchilin, and L. Huang. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. *Biochim. Biophys. Acta* **1062**:142–148 (1991).
22. M. S. Wu, J. C. Robbins, R. L. Bugianesi, M. M. Ponpipom, and T. Y. Shen. Modified in vivo behavior of liposomes containing synthetic glycolipids. *Biochim. Biophys. Acta* **674**:19–29 (1981).
23. D. Fisher. The separation of cells and organelles by partitioning in two-polymer aqueous phases. *Biochem. J.* **196**:1–10 (1981).
24. A. A. Gabizon. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res.* 52:891–896 (1992).
25. D. Papahadjopoulous, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K.-D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* 88:11460–11464 (1991).
26. C. Tagesson, O. Stendahl, and K.-E. Magnusson. The clearance of liposomes after intravenous injection into mice in relation to their physico-chemical properties as assessed by partitioning in an aqueous biphasic system. *Studia Biophys* **64**:151–160 (1977).
27. A. Supersaxo, W. Rubas, H. R. Hartmann, H. Schott, H. Hengartner, and R. A. Schwendener. The antitumor effect of lipophilic derivatives of 5-fluoro-2′-deoxyuridine incorporated into liposomes. *M. Microencapsul.* 5:1–11 (1988).
28. M. van Borssum Waalkes and G. L. Scherphof. Liposome-incorporated 3′,5′-*o*-depalmitoyl-5-fluoro-2′-deoxyuridine as a slow-release anti-tumor drug depot in rat liver macrophages. *Select. Cancer Ther.* 6:15–22 (1990).
29. K. Maruyama, A. Mori, S. J. Kennel, M. van Borssum Waalkes, G. L. Scherphof, and L. Huang. Drug delivery by organ-specific liposomes. In R. L. Dunn (ed.), *Polymeric Drugs and Drug Delivery Systems,* American Chemical Society, 1991, pp. 275–284.
30. T. M. Allen, P. Williamson, and R. A. Schegel. Phosphatidylserine as a determinant of reticuloendothelial recognition of liposome models of the erythrocyte surface. *Proc. Natl. Acad. Sci. USA* 85:8067–8071 (1988).