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Biodistribution and immunotargetability of ganglioside-stabilized dioleoylphosphatidylethanolamine liposomes

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The biodistribution and immunotargetability of liposomes composed primarily of dioleoylphosphatidylethanolamine (DOPE) or dioleoylphosphatidylcholine (DOPC) in mice injected via the tail vein were examined and compared. The ganglioside G_{M1} (7 mol%) prolonged the circulation of DOPC but not DOPE liposomes. Gangliosides G_{D1a} and G_{T1b} (7 mol%) also increased the amount of DOPC liposomes remaining in circulation, and to a similar extent as G_{M1}, at 15 min post injection. However, these liposomes were cleared from the circulation by 2.5 h. Monoclonal antibody 34A, which specifically binds to a surface glycoprotein (gp 112) of the pulmonary endothelial cell surface, was coupled with *N*-glutarylphosphatidylethanolamine and incorporated into liposomes by a dialysis procedure. These 34A-immunoliposomes, composed of DOPE and G_{M1} (7 mol%), but not the antibody-free liposomes, accumulated efficiently (≈ 24% of the injected dose) in the lungs. Inclusion of cholesterol (31 mol%) enhanced the lung accumulation of both DOPE/G_{M1} immunoliposomes and DOPC/G_{M1} immunoliposomes to 33% and 51% of the injected dose, respectively. The transient increase in DOPC liposome circulation provided by G_{D1a} and G_{T1b} was sufficient to enhance DOPC immunoliposome binding, where 44% and 43% of the injected dose of DOPC/Chol/G_{D1a} and DOPC/Chol/G_{T1b} immunoliposomes accumulated in lung at 15 min after injection, respectively. In general, cholesterol-containing DOPC liposomes were more targetable than DOPE liposomes, and the degree to which these liposomes avoid RES uptake influences their targetability. The results presented here are relevant to the design of targetable drug delivery vehicles.

Introduction

The use of liposomes for drug delivery is a topic of intense investigation. We have recently described a system using monoclonal antibodies to target liposomes to murine lung *in vivo* [1–4]. These immunoliposomes have incorporated monoclonal antibody 34A to gp112, a pulmonary endothelial cell antigen located abundantly on the lumenal surface of the pulmonary capillary vessel wall [5,6]. Several physical parameters of the 34A-immunoliposomes have been investigated to determine their effects on target binding. These parameters have included antibody/lipid ratio, immunoliposome size, injection dose, and lipid composition [3,4]. Under optimal conditions, approx. 70% of the injected dose accumulated in lung [3].

All previous work was done with liposomes composed primarily of EPC. The present studies described here investigate the targeting of 34A-immunoliposomes composed primarily of DOPE. DOPE is a major functional component of pH-sensitive and target-sensitive immunoliposomes. pH-sensitive immunoliposomes deliver entrapped contents to the cytoplasm of the target cells [7–12]. Target-sensitive immunoliposomes become destabilized and release entrapped contents upon binding to the target cell surface [13,14]. While delivery of

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Abbreviations: Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DTPA-SA, diethylenetriaminepentaacetic acid stearylamide; EPC, egg phosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt; NGPE, *N*-glutarylphosphatidylethanolamine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RES, reticuloendothelial system (also named as mononuclear phagocyte system).

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antitumor [9] and antiviral [14] drugs, toxin [11], and DNA [12] has been well documented *in vitro*, very few studies have been done *in vivo* [10,15].

DOPE has a propensity to form the nonbilayer hexagonal (H_{II}) phase under conditions of physiological temperature and pH [16]. The dynamic shape concept developed by Cullis and De Kruijff [16], and expanded by Israelachvili et al. [17], proposes that the dynamic molecular shape of the lipid determines the equilibrium configuration upon lipid aggregation. DOPE, a cone-shaped molecule, can be complemented with an inverted cone-shaped molecule to form a stable bilayer structure [16–18]. Acidic amphiphiles and acylated antibody are such DOPE bilayer stabilizers for pH-sensitive and target-sensitive immunoliposomes, respectively. The gangliosides G_{M1} [19] and G_{D1a} [20] have also proven to be potent DOPE bilayer stabilizers. Inclusion of G_{M1}, G_{D1a}, or G_{T1b} in EPC or EPC/cholesterol (Chol) liposomes greatly reduced lysis and content leakage upon incubation in human plasma [21]. RES uptake of liposomes has been a barrier for targeting liposomes to tumors or organs other than liver and spleen. Inclusion of G_{M1} in a wide variety of liposome compositions [22,23], including pH-sensitive liposomes [24], prolongs liposome circulation half-lives in mice by reducing RES uptake. The effect of reducing RES uptake on targetability was revealed when 34A-immunoliposomes composed of EPC/Chol/G_{M1} showed higher levels of lung binding and retention than immunoliposomes composed of EPC/Chol/phosphatidylserine (PS) [3], where it is well established that PS elevates liposome uptake by the RES [25]. Therefore, G_{M1} appeared to retain the ability of reducing RES uptake in targeted PC immunoliposomes, and was thus chosen as the stabilizer for the targeted DOPE immunoliposomes described here. The biodistribution of antibody-free liposomes of this composition was also investigated. A comparison with G_{D1a} and G_{T1b}-stabilized DOPE liposomes and immunoliposomes as well as with ganglioside-containing DOPC liposomes and immunoliposomes is also presented. The role of Chol in liposome biodistribution and targetability was also examined.

Materials and Methods

Materials

DOPE, DOPC, and EPC were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and Chol was purchased from Sigma Chemical Co. (St. Louis, MO). Gangliosides G_{M1}, G_{D1a}, and G_{T1b} were purchased from Matreya, Inc. (Pleasant Gap, PA). ^{111}In and ^{125}I were from New England Nuclear. All other chemicals were of reagent grade. *N*-Glutarylphosphatidylethanolamine (NGPE) was synthesized as described [26] with modifications. Briefly, DOPE was combined with glutaric anhydride and triethylamine in $CHCl_3$ at a molar ratio of 1:3:1 (DOPE/glutaric anhydride/triethylamine). The mixture was incubated at 20°C for approximately 4 h. After this time, the reaction mixture no longer stained positively with ninhydrin. The mixture was then subjected to water extraction followed sequentially by dialysis against 0.01 M sodium borate buffer, pH 8.5, and ddH_2O to remove triethylamine and byproduct glutaric acid. Phospholipid and NGPE purity was checked by thin-layer chromatography, using $CHCl_3/CH_3OH/ddH_2O$ (55:45:10, v/v) as the solvent system, followed by I_2 vapor detection. Phospholipid and NGPE concentrations were determined by phosphate assay [27]. Diethylenetriaminepentaacetic acid stearylamide (DTPA-SA) was synthesized as described [28]. ^{111}In-labeled DTPA-SA (^{111}In-DTPA-SA) was prepared as described [3]. This lipophilic radiolabel is not transferred to serum components from liposomes (unpublished data), and is not rapidly metabolized *in vivo* [2].

Antibody preparation and conjugation

Rat monoclonal antibody 34A (IgG_{2a}) was purified from *nu/nu* mouse ascites fluid as described [29]. 34A was radiolabeled with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL), and purified using a Bio-Gel P-4 (Bio-Rad, Richmond, CA) spin column. Conjugation of 34A with NGPE was performed as described [3]. Briefly, NGPE dissolved in $CHCl_3$ was dried with N_2 gas, vacuum desiccated for approximately 0.5 h, and resuspended in Mes buffer (5 mM Mes, 150 mM NaCl (pH 5.0)) containing 0.15 M octyl β-D-glucopyranoside (OG) (NGPE to OG molar ratio = 1:16). 0.25 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.10 M *N*-hydroxysulfosuccinimide (S-NHS) in ddH_2O were then added to a molar ratio of 1:55:22 (NGPE/EDC/S-NHS) and incubated at room temperature for 10 min. The pH of the mixture was then adjusted to pH 7.8 with 0.1 M Hepes buffer (pH 7.8) and 1 M NaOH. 34A and a trace amount of ^{125}I-labeled 34A were then added to a molar ratio of 1:0.05 (NGPE/antibody) and incubated at 4°C for 8 h with gentle stirring.

Immunoliposome preparation

Immunoliposomes containing NGPE-conjugated 34A were prepared by a detergent dialysis method as described [2]. Antibody-free liposomes were prepared by the same method without the addition of derivitized antibody. Briefly, lipid mixtures containing trace amounts of ^{111}In-DTPA-SA were dried from organic solvent with N_2 gas and vacuum desiccated for 2 h. The dried lipid films were solubilized upon addition of 0.15 M OG in PBS, pH 7.5 (lipid/OG molar ratio = 1:9) and the conjugation reaction mixture containing derivitized 34A. The resulting solution (lipid/antibody

weight ratio = 3:1) was vortexed vigorously and dialyzed against PBS (pH 7.5) for 36 h at 4°C. The immunoliposomes were extruded three times through a stack of two 0.6 μm pore size Nuclepore membranes and six times through a stack of two 0.2 μm pore size membranes to generate immunoliposomes with a diameter of less than 200 nm. The average size of the immunoliposomes was measured using a Coulter N4SD submicron particle size analyzer (Hialeah, FL). Unincorporated antibody was separated from the immunoliposomes using a Bio-Gel A15M (Bio-Rad) column. The final weight ratio of incorporated antibody to lipid was determined from the radioactivities of ^{125}I and ^{111}In, respectively. Immunoliposomes were diluted to 120 μg lipid/200 μl PBS (pH 7.5).

Biodistribution studies

Liposomes or 34A-immunoliposomes (120 μg of lipid) were injected into male Balb/c mice (6–8 weeks old) via the tail vein. At the desired time interval, the mice were lightly anesthetized, weighed, and bled by retro-orbital puncture. The mice were killed by cervical dislocation and dissected. In addition to blood, organs were collected, weighed, and analyzed for ^{111}In radioactivity in a Beckman 5500B gamma counter. The results are presented as percent of the total injected dose for each organ. The total radioactivity in blood was determined by assuming that the total blood volume was 7.3% of the body weight [30]. Blood correction factors for each organ were determined using ^{51}Cr-labeled red blood cells. Briefly, red blood cells were isolated from male Balb/c mice of the same age and weight, washed with sterile isotonic saline solution (Curtis Matheson Sci., Inc., Marietta, GA), and labeled with ^{51}Cr at 37°C. Mice were injected with labeled cells ($8 \cdot 10^7$ red blood cells in 200 μl saline solution) and killed 30 min post injection. Lung, liver, and spleen contained approx. 7%, 9%, and 1% of the total blood, respectively.

Results

Biodistribution studies of ganglioside-containing antibody-free liposomes

Antibody-free liposomes composed of DOPC (DOPC-based) and DOPE (DOPE-based), with or without Chol, and containing 7 mol% ganglioside G_{M1}, G_{Dla}, or G_{T1b} were injected by means of the tail vein. The biodistribution of the injected ^{111}In-DTPA-SA labeled liposomes among various major organs was measured at different time periods. For all lipid compositions examined, greater than 90% of the recovered ^{111}In radioactivity was distributed among the blood (Fig. 1), liver (Fig. 2), and spleen (Fig. 3) for each time point. Liposomes composed of DOPC/Chol (67:33 molar ratio) showed rapid clearance from circulation

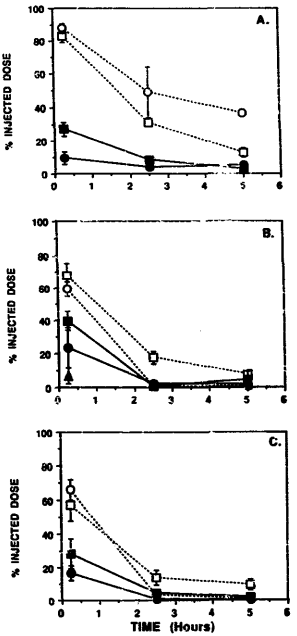

Fig. 1. Circulation of DOPE and DOPC liposomes, with or without Chol, and containing ganglioside G_{M1}, G_{Dla}, or G_{T1b}. The molar ratio of liposomes without Chol was 93:7 (phospholipid/ganglioside) and with Chol was 62:31:7 (phospholipid/Chol/ganglioside). Liposomes (120 μg of lipid) labeled with ^{111}In-DTPA-SA were injected i.v. Percent injected dose in blood was measured at the indicated time intervals. Bars represent S.D. ($n = 3$). (A) DOPC/G_{M1}, 92 nm (□); DOPC/Chol/G_{M1}, 189 nm (○); DOPE/G_{M1}, 168 nm (■), and DOPE/Chol/G_{M1}, 178 nm (●). (B) DOPC/G_{Dla}, 97 nm (□); DOPC/Chol/G_{Dla}, 165 nm (○); DOPE/G_{Dla}, 135 nm (■); DOPE/Chol/G_{Dla}, 158 nm (●), and DOPC/Chol, 200 nm (▲). (C) DOPC/G_{T1b}, 80 nm (□); DOPC/Chol/G_{T1b}, 159 nm (○); DOPE/G_{T1b}, 169 nm (■), and DOPE/Chol/G_{T1b}, 135 nm (●).

by the RES, predominantly by liver (Figs. 1B, 2B, and 3B). Addition of G_{M1} to DOPC/Chol liposomes (DOPC/Chol/G_{M1}, 62:31:7) greatly prolonged the liposome residence in circulation (Fig. 1A). Inclusion of Chol has been shown to increase liposomal half-lives in the bloodstream [31,32] possibly by making the lipo-

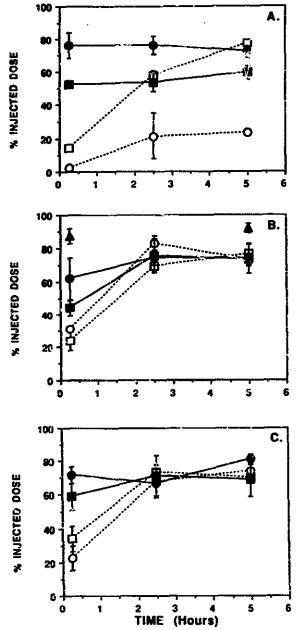

Fig. 2. Liver uptake of DOPE and DOPC liposomes, with or without Chol, and containing ganglioside G_{M1}, G_{D1a}, or G_{T1b}. Liposomes (120 μg of lipid) labeled with ^{111}In-DTPA-SA were injected i.v. Percent injected dose in liver was measured at the indicated time intervals. Bars represent S.D. ($n = 3$). The symbols in A, B, and C are as indicated in Fig. 1

some less susceptible to destruction by various serum components (for review, see Ref. 33) and/or by decreasing phagocytic uptake by hepatic Kupffer cells [34–36]. In agreement with these findings, the presence of Chol in the liposome composition enhanced the circulation half-life in comparison with DOPC/G_{M1} (93:7) liposomes (Fig. 1A). It has previously been demonstrated that the liver accumulates Chol-poor liposomes more than Chol-rich liposomes, whereas spleen uptake of Chol-rich liposomes is greater than that of Chol-poor liposomes [34,35]. In the present studies of ganglioside-containing PC liposomes, the liver showed a greater affinity for the Chol-free DOPC/G_{M1} liposomes (Fig. 2A) while the spleen demonstrated greater uptake of the DOPC/Chol/G_{M1} liposomes (Fig. 3A). This differentiation was especially evident by 2.5 h. The gangliosides G_{D1a} and G_{T1b} enhanced the amount of DOPC-based liposomes in the blood, regardless of the presence of Chol, at 15 min post injection (Fig. 1B and 1C) in comparison with the DOPC/Chol liposomes. The effect of G_{D1a} and G_{T1b}, present at 7 mol%, on liposome circulation at 15 min was slightly less than that of G_{M1}, and these liposomes were cleared from blood by 2.5 h. The spleen contin-

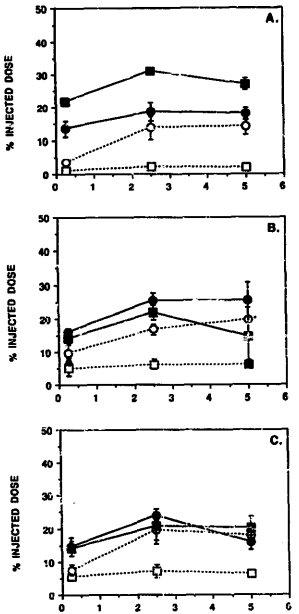

Fig. 3. Spleen uptake of DOPE and DOPC liposomes, with or without Chol, and containing ganglioside G_{M1}, G_{D1a}, or G_{T1b}. Liposomes (120 μg of lipid) labeled with ^{111}In-DTPA-SA were injected i.v. Percent injected dose in spleen was measured at the indicated time intervals. Bars represent S.D. ($n = 3$). The symbols in A, B, and C are as indicated in Fig. 1.

ued to show a greater uptake of Chol-containing liposomes (Figs. 3B and 3C) while the liver uptake of such liposomes was similar regardless of the presence of Chol (Figs. 2B and 2C). Indeed, when G_{M1} was substituted with G_{D1a} or G_{T1b}, the uptake by liver was so enhanced by 2.5 h that any differentiation for Chol was no longer observed. Thus G_{M1} may have sufficiently suppressed liver uptake for the Chol differentiation to be revealed. Alternatively, the process by which Chol decreases liver uptake of DOPC liposomes may have been preserved upon the addition of G_{M1}, but not of G_{D1a} or G_{T1b}.

The biodistribution of DOPE-based liposomes differed greatly from the corresponding DOPC-based liposomes. The amounts of liposomes composed of DOPE/G_{M1} (93:7) and DOPE/Chol/G_{M1} (62:31:7) in blood were significantly higher than DOPC/Chol liposomes yet greatly reduced from their DOPC-based counterparts at 15 min (Fig. 1A). By 2.5 h both liposome compositions were removed from the circulation. Thus the activity of G_{M1} may be influenced by the surrounding lipid matrix or environment. Substitution of G_{D1a} or G_{T1b} for G_{M1} in both liposome compositions produced similar results (Figs. 1B and 1C). Liposome preparations containing DOPE/Chol (67:33) showed massive aggregation following dialysis, likely indicating the predominant presence of DOPE in the nonbilayer H_{II} phase.

Interestingly, Chol reduced the amounts of DOPE-based liposomes in blood at 15 min (Figs. 1A, 1B, and 1C). Furthermore, by 15 min after injection, the liver showed greater uptake of DOPE/Chol/G_{M1} liposomes than the Chol-free DOPC/G_{M1} liposomes (Fig. 2A), while the spleen demonstrated greater uptake of the Chol-free DOPE/G_{M1} liposomes (Fig. 3A). Liver uptake of G_{D1a} or G_{T1b}-stabilized DOPE-based liposomes was nondiscriminant between Chol-containing and Chol-free liposomes (Figs. 2B and 2C). Spleen uptake of DOPE-based liposomes, though greater in all cases compared to the DOPC-based liposomes, was similar for the DOPE/Chol/G_{D1a} or DOPE/Chol/G_{T1b} liposomes and the Chol-free DOPE/G_{D1a} or DOPE/G_{T1b} liposomes (Figs. 3B and 3C). This may suggest that G_{M1} influences the DOPE-based liposome uptake and Chol discrimination by liver and spleen differently than G_{D1a} or G_{T1b}.

Thus, all three gangliosides increased the amount of antibody-free, DOPC-based liposomes remaining in circulation at 15 min post injection. Substitution of DOPC for DOPE significantly reduced the amounts of liposomes in the circulation at this time interval, and enhanced spleen uptake.

Lung targeting of G_{M1}-containing 34A-immunoliposomes

The lung accumulation of DOPE-based and DOPC-based, G_{M1}-containing 34A-immunoliposomes at 15 min post injection was examined and the results are presented in Table I. It has been well established that the equilibrium phase of unsaturated PE, such as DOPE, at physiological temperature and pH is the nonbilayer H_{II} phase [16]. A mouse monoclonal IgG_{2a} antibody has previously been derivitized with palmitic acid and the resulting palmitoyl-IgG_{2a} conjugate (**pIgG**) alone stabilized the bilayer phase of DOPE at a minimal pIgG to DOPE molar ratio of $2.5 \cdot 10^{-4}$ [37]. However, this stabilization activity by derivitized antibody alone has so far only been observed with this particular IgG_{2a}. Dioleoylphosphatidic acid was later included in this immunoliposome formulation for improved stability (Pinnaduwage and Huang, personal communication). The DOPE-based 34A-immunoliposomes in the present investigations included the gangliosides G_{M1}, G_{D1a}, and G_{T1b} as stabilizers. DOPE/G_{M1} (93:7) as well as DOPC/G_{M1} (93:7) antibody-free

TABLE I

Effect of immunoliposome composition on biodistribution [a]

Lipid composition	Antibody/lipid (w/w)	Diameter [b] (nm)	Percent injected dose [c]			
			lung	blood	liver	spleen
EPC/Chol (75:25)	1:6.4	160	33.9 (2.1)	15.2 (1.5)	25.3 (1.9)	4.0 (0.7)
DOPC/G_{M1} (93:7)	1:6.0	138	11.1 (3.9)	44.0 (5.1)	15.0 (2.8)	1.2 (0.8)
DOPC/G_{M1} (93:7)	0	89	0.0 (0.0)	73.1 (8.1)	13.0 (4.1)	0.4 (0.3)
DOPE/G_{M1} (96:4)	1:6.9	157	16.8 (0.8)	23.7 (2.0)	29.1 (0.9)	4.5 (0.3)
DOPE/G_{M1} (93:7)	1:7.5	153	24.0 (1.5)	20.8 (3.7)	27.5 (0.7)	6.0 (0.7)
DOPE/G_{M1} (90:10)	1:7.8	147	25.1 (5.8)	16.3 (3.7)	27.8 (3.0)	4.5 (0.9)
DOPE/G_{M1} (93:7)	0	126	0.0 (0.0)	26.6 (8.6)	47.0 (5.0)	11.3 (3.6)
DOPC/Chol/G_{M1} (62:31:7)	1:5.8	132	41.5 (0.5)	19.6 (0.6)	20.1 (1.2)	3.3 (0.6)
DOPE/Chol/G_{M1} (62:31:7)	1:6.9	183	33.2 (2.5)	2.7 (0.2)	54.4 (3.0)	4.7 (0.6)

[a] Immunoliposomes (120 μg of lipid) of the indicated lipid composition, size, and antibody content, and labeled with ^{111}In-DTPA-SA were injected i.v.

[b] Immunoliposomes of diameter < 200 nm were generated by extrusion following dialysis.

[c] Percent injected dose ± S.D. (n = 3) per organ was measured 15 min after injection.

liposomes showed no accumulation in lung. However, DOPE/G_{M1} 34A-immunoliposomes, with the amount of ganglioside varying from 4 to 10 mol%, significantly accumulated in lung. Varying the amount of G_{M1} in the immunoliposome composition revealed a saturation effect at 7 mol%, where 24% of the injected dose was present in lung. The half-life of retention of these immunoliposomes in lung was approx. 3 h (data not shown).

The effect of Chol on liposome targetability was also examined (Table I). DOPC/G_{M1} (93:7) and DOPE/G_{M1} (93:7) immunoliposomes revealed lung accumulations of 11% and 24% of the injected dose, respectively. Inclusion of Chol in the lipid compositions increased lung binding to 42% and 33% of the injected dose for DOPC/Chol/G_{M1} (62:31:7) and DOPE/Chol/G_{M1} (62:31:7) immunoliposomes, respectively.

Therefore, the results reveal that 7 mol% G_{M1} is sufficient for optimal DOPE-based 34A-immunoliposome lung targeting. Furthermore, the addition of Chol enhanced targeting of both DOPC-based and DOPE-based immunoliposomes, with a more dramatic enhancement observed for the former.

Lung targeting of G_{D1a} or G_{T1b}-containing 34A-immunoliposomes

The effect of substituting G_{M1} with G_{D1a} or G_{T1b} in the 34A-immunoliposome compositions on lung targeting was examined, and the results are presented in Fig. 4. DOPC/Chol/G_{D1a} (62:31:7) immunoliposomes revealed comparable lung accumulation to DOPC/Chol/G_{M1} (62:31:7) immunoliposomes (Fig. 4B). 44% and 51% of the injected dose, respectively, were present in lung at 15 min post injection. The presence of either ganglioside in the lipid composition enhanced lung binding in comparison with DOPC/Chol (67:33) immunoliposomes, where 29% of the injected dose accumulated in lung. Furthermore, Chol-free DOPC/G_{M1} (93:7) and DOPC/G_{D1a} (93:7) immunoliposomes showed comparable lung binding with 18% and 13% of

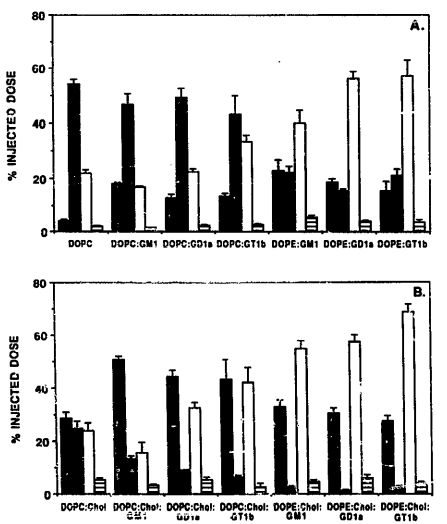

Fig. 4. Biodistribution of DOPC and DOPE 34A-immunoliposomes, without (A) or with (B) Chol, and containing ganglioside G_{M1}, G_{D1a}, or G_{T1b}. Lipid molar ratio: DOPC/Chol, 67:33; phospholipid/ganglioside, 93:7; phospholipid/Chol/ganglioside, 62:31:7. Immunoliposomes (120 μg of lipid, 27–37 μg of antibody, 150–250 nm diameter) labeled with ^{111}In-DTPA-SA were injected i.v. Percent injected dose in lung (■), blood (▨), liver (□), and spleen (▤) were measured 15 min after injection. Bars represent S.D. ($n = 3$).

the injected dose, respectively, accumulating in lung (Fig. 4A). The presence of either ganglioside in the Chol-free immunoliposomes enhanced lung accumulation as only 4% of the injected dose of purely DOPC immunoliposomes was present in lung. The substitution of G_{M1} with G_{T1b} also did not dramatically affect lung accumulation at 15 min post injection. 43% and 14% of the injected dose of DOPC/Chol/G_{T1b} (62:31:7) and DOPC/G_{T1b} (93:7) immunoliposomes, respectively, accumulated in lung.

Substitution of G_{M1} with G_{D1a} or G_{T1b} in the DOPE-based immunoliposomes also did not dramatically affect lung accumulation. 33%, 31%, and 28% of the injected dose of DOPE/Chol/G_{M1}, DOPE/Chol/G_{D1a}, and DOPE/Chol/G_{T1b} (DOPE/Chol/ganglioside, 62:31:7) immunoliposomes, respectively, were present in lung at 15 min post injection (Fig. 4B). 24%, 19%, and 16% of the injected dose of Chol-free DOPE/G_{M1}, DOPE/G_{D1a}, and DOPE/G_{T1b} (DOPE/ganglioside, 93:7) immunoliposomes, respectively, accumulated in lung (Fig. 4A). Massive aggregation following dialysis of DOPE/Chol (67:33) with derivitized 34A (antibody/lipid weight ratio = 1:3) was observed which again indicated the presence of DOPE in the H_{II} phase.

Thus, the substitution of G_{M1} with G_{D1a} or G_{T1b} did not dramatically reduce the targetability of DOPC-based or DOPE-based immunoliposomes. The presence of Chol enhanced the targetability of all immunoliposome preparations. Overall, Chol-containing DOPC-based immunoliposomes are more targetable than the DOPE-based immunoliposomes.

Discussion

Liposomes removed from the circulation accumulate in the RES, with liver and spleen as the sites of greatest uptake. While RES uptake has been shown to be influenced by vesicle size [38] and charge [39], two parameters have been proposed to account overall for differences in the RES uptake. They are liposome hydrophobicity and susceptibility to opsonization. The two properties may not be mutually exclusive, as it has been suggested that opsonins responsible for enhanced RES uptake prefer to associate with hydrophobic surfaces [33]. Indeed, several studies suggest RES uptake increases with particle hydrophobicity. The clearance of poloxamer-coated polystyrene microspheres from the circulation, and their accumulation in liver and spleen, decreased with increased particle surface hydrophilicity [40,41]. The reason for an apparent relationship between hydrophobicity and RES uptake, however, remains unclear.

The present studies in general support the hydrophobic model of particle uptake. A repulsive force caused by the work of dehydration of interacting hydrophilic surfaces was measured for various bilayer compositions, and this hydration repulsive force was found to be greater for PC versus PE bilayers [42]. Therefore, substitution of DOPC with DOPE likely decreased the strength of bilayer hydration which in turn may have increased liposome clearance. A greater hydration repulsive force or water barrier of the PC liposome surface may reduce opsonin association and/or RES phagocytic cell interaction. The large hydrated ganglioside headgroups [43] would be expected to increase the surface hydrophilicity of phospholipid bilayers. Therefore, the results presented in which G_{M1} prolonged DOPC-based liposome circulation agree with the hydrophobicity model. Substitution of G_{M1} with G_{D1a} or G_{T1b} would increase the amount of sialic acid content and would be expected to further increase the liposomal surface hydrophilicy. However, the reasons for the failure of these gangliosides to further increase liposome circulation is at present unclear.

The susceptibility of liposomes to opsonization by plasma proteins and subsequent binding and uptake by RES cells may be related to bilayer rigidity. Rigid liposome formulations are more resistant to phospholipid exchange with HDL [44], and display elevated levels in circulation [45]. Inclusion of Chol in PC liposomes tightens the phospholipid packing [46] and prevents destabilization caused by insertion of HDL-associated apolipoproteins and subsequent lipid transfer [47]. Inclusion of G_{M1} in PC/Chol liposomes further reduces lipid transfer with HDL [23]. Gangliosides also decreased plasma-induced content leakage synergistically with Chol [21]. Our results support this model in that G_{M1} enhanced DOPC/Chol liposome circulation, and DOPC/Chol/G_{M1} liposomes remained in circulation longer than Chol-free DOPC/G_{M1} liposomes. G_{D1a} and G_{T1b} may also have reduced opsonization and subsequent RES uptake, yet to a much lesser extent. According to this model, substitution of DOPC with DOPE may render the liposomes more susceptible to opsonization as observed by reduced circulation.

In vitro studies suggest that serum contains organ-specific opsonins [48]. Liver-specific opsonin enhances phagocytosis of Chol-poor liposomes by Kupffer cells, while spleen-specific opsonin enhances phagocytosis of Chol-rich liposomes by spleen macrophages [48]. Furthermore, liver-specific opsonin(s) appeared to have a reduced affinity for rigid liposome compositions [49]. Increasing liposome hydrophobicity enhanced spleen macrophage uptake, possibly as a result of increased spleen-specific opsonin affinity [49]. According to this model, our results would suggest that G_{M1}, and to a lesser extent G_{D1a} and G_{T1b}, may have reduced liver-specific opsonin(s) affinity and subsequent Kupffer cell phagocytosis. Substitution of DOPC with DOPE may

have further modified opsonin affinity, and this increase in liposome hydrophobicity may account for the enhanced spleen uptake. The existence and characterization of organ-specific opsonins *in vivo* await elucidation.

The rapid clearance of liposomes from the circulation by the RES is an obstacle for the targeting of liposomes to tumors or to organs other than liver and spleen. Our laboratory has recently demonstrated that 34A-immunoliposome formulations which reduce RES uptake allow enhanced lung target binding [3]. The present results further examine this relationship between prolonged liposome circulation and targetability. DOPC/Chol/G_{M1} liposomes circulate longer than DOPC/Chol or DOPE-based liposomes, and in turn demonstrated greater target binding. Inclusion of G_{D1a} and G_{T1b} also increased the amount of DOPC/Chol liposomes in blood at 15 min post injection, with reduced liver uptake. Kinetic binding studies using our model system suggest that target binding is rapid, reaching steady state within 5 min [3]. Thus the effect of G_{D1a} and G_{T1b} on liposome circulation was within this short time period and allowed enhanced target binding. The relationship between liposome circulation and target binding is less clear among the Chol-free liposomes, and such targetability may reflect other influencing factors. Results from our 34A-immunoliposome model system may differ from other systems, for example tumor models with low vascularization, reduced binding affinity, and/or reduced antigen expression, where prolonged circulation by G_{D1a} and G_{T1b} may not be sufficient for increased target binding.

Immunoliposome stability is also important for target binding. As noted above, Chol prevents PC liposome destabilization by HDL. At high concentrations, Chol also stabilizes PE liposomes [50]. Inclusion of Chol in large DOPE/OA liposomes prepared by dialysis was shown to reduce content leakage upon incubation in human plasma [51]. The present studies demonstrate that 31 mol% Chol enhances the binding of all DOPC-based and DOPE-based immunoliposome formulations. Chol may prevent interaction with proteins which destabilize the immunoliposomes or sterically hinder target binding.

In summary, we have described the biodistribution properties of ganglioside-stabilized DOPE-based liposomes in comparison with ganglioside-containing DOPC-based liposomes. A positive correlation between liposome circulation and targetability was revealed among Chol-containing liposomes. Furthermore, Chol increases liposome targetability for both DOPE-based and DOPC-based liposomes. DOPE plays a major functional role in target-sensitive and pH-sensitive immunoliposomes. Thus, the immunoliposome parameters studied here should prove useful in the design of drug carriers which release their contents at or near the target cell surface or to the target cell cytoplasm *in vivo*.

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