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Rapid report

Some negatively charged phospholipid derivatives prolong the liposome circulation in vivo

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A series of negatively charged phospholipid derivatives has been synthesized by coupling aliphatic dicarboxylic acids, $HOOC(CH_2)_nCOOH$, to dioleoylphosphatidylethanolamine (DOPE). The individual derivatives were incorporated into egg phosphatidylcholine/cholesterol liposomes (2 : 1, molar ratio) and injected into mice to test its effect on liposome circulation in vivo. The effectiveness of DOPE derivatives was dependent on the hydrocarbon chain length between the terminal carboxyl group and the amide bond. *N*-Glutaryl DOPE and *N*-adipyl DOPE were effective in prolonging the circulation time of liposomes. On the other hand, liposome uptake by the liver and spleen was increased by the addition of *N*-malonyl DOPE or *N*-succinyl DOPE, while it was not changed by the addition of *N*-pimelyl DOPE and *N*-suberyl DOPE. Our observation suggested that not all negatively charged phospholipids enhance liposome uptake by RES, some even reduce the uptake.

Clearance of liposomes from circulation in vivo depends on the rate of uptake by RES cells in liver and spleen [1–5]. The rate of liposome uptake by the RES is believed to be related to the process of opsonization (for a review, see Ref. 6) or dysopsonization of liposomes [7]. There are two different ways with which liposomes may interact with macrophages. Liposomes may directly interact with the surface receptor(s) of macrophages, or indirectly via certain serum proteins. However, the diversity and complexity of the opsonization process of liposome make it difficult to assess the role of various proteins and lipids in the RES uptake of liposomes. It has been observed that liposomes containing negatively charged phospholipids are removed more rapidly from the circulation and localized mainly in the liver and spleen [8–11]. Phosphatidylserine (PS), phophatidylglycerol (PG), and other negatively charged phospholipids are known to enhance liposome uptake by the RES cells [11,12]. However, ganglioside G_{M1}, sulfatides, and phosphatidylinositol (PI), which are also negatively charged, inhibit the RES uptake and prolong the circulation time of liposomes [2,3]. It has been proposed that the negatively charged carboxyl group of G_{M1} which is shielded by a bulky, neutral, hydrophilic sugar moiety may contribute to RES avoidance by decreasing or preventing opsonization, i.e., the 'shielded negative charge' hypothesis [2,3].

In this study we prepared liposomes containing negatively charged synthetic phospholipid derivatives and assayed the biodistribution of these liposomes in mice. We found that some of the phospholipid derivatives showed the ability of prolonging the liposome circulation time, yet others showed an opposite activity of enhancing the uptake by the RES. These unusual findings have left questions on the generalized idea about the fate of negatively charged liposomes in vivo and the validity of the 'shielded negative charge' hypothesis.

Some of the DOPE derivatives were prepared by coupling dicarboxyl ligands to DOPE via dicyclohexylcarbodiimide (DCC) activation [13]. DOPE in dry ethylacetate was mixed with malonic acid, adipic acid, pimelic acid, or suberic acid. The mixtures were incubated at 45°C for 4 h. The final molar ratio of DOPE, ligand, and DCC was 1 : 1.2 : 1.3. The conversion yield was approx. 60% after 4 h incubation. The rest of

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Abbreviations: DCC, dicyclohexylcarbodiimide; DOPE, dioleoylphosphatidylethanolamine; DTPA-SA, diethylenetriaminepentaacetic acid distearylamine complex; G_{M1}, monosialoganglioside, II^{3}Neu5Ac-GgOse$_4$Cer; NAPE, *N*-adipyl DOPE; NGPE, *N*-glutaryl DOPE; NMPE, *N*-malonyl DOPE; NPPE, *N*-pimelyl DOPE; NSPE, *N*-succinyl DOPE; NSBPE, *N*-suberyl DOPE; PC, phosphatidylcholine; PI, phosphatidylinositol; RES, reticuloendothelial system.

DOPE derivatives were prepared from anhydrides of dicarboxylic acid and DOPE as previously described [14]. DOPE in dry chloroform was mixed with succinic anhydride or glutaric anhydride and incubated in the presence of triethylamine at room temperature for 4 h. The final molar ratio of DOPE, ligand, and triethylamine was 1:2:1. The conversion yield of each reaction was over 95%. The reaction progress was confirmed by thin-layer chromatography (TLC) with a solvent system of $CHCl_3/MeOH/H_2O$ (70:30:5, v/v). The final reaction mixture was dried in a rotatory evaporator and then dissolved in methanol. The lipid solution was applied to DEAE-Sephadex which was pretreated with 0.5 M ammonium acetate in methanol and equilibrated with methanol. The DOPE derivatives were eluted by a gradient of ammonium acetate 0 to 0.2 M and fractions containing DOPE derivatives were extensively dialyzed against water and lyophilized. Phosphate assay was performed for quantitation of DOPE derivatives [15]. Partial characterization of DOPE derivatives was done by HPTLC and infrared spectroscopy. The purified DOPE derivatives appeared to be single spots on HPTLC plate with the solvent system mentioned above. R_f values of DOPE derivatives were 0.24, 0.26, 0.27, 0.29, 0.30, 0.31, and 0.51 for NMPE, NSPE, NGPE, NAPE, NPPE, NSBPE, and DOPE, respectively. The appearance of O-H stretching of carboxylic acid was indicated by infrared absorbance at 3000 cm^{-1}.

Liposomes were prepared from egg PC, cholesterol, and each DOPE derivative (10:5:1, molar ratio) with a tracer marker ^{111}In-DTPA-SA. The synthesis of DTPA-SA has been described [16]. The ^{111}In-DTPA-SA was prepared by mixing 1 μl of $^{111}InCl_3 \cdot HCl$ with 600 μl of 1 mM DTPA-SA in ethanol at 80°C with brief sonication. Lipids were dried under a stream of N_2 gas and then vacuum-dessicated for over 1 h to remove any trace of organic solvent. PBS (pH 7.4) was added and the lipid mixture was allowed to hydrate for 1 h at room temperature. After rigorous vortexing the suspension was extruded through two stacked Nuclepore polycarbonate filter (five times through filters of 0.4 μm pore size and ten times through filters of 0.2 μm pore size). Liposome diameter measured by a Coulter N4SD submicron particle analyzer (Hialeah, FL) was approx. 175 nm.

^{111}In-labeled liposomes (0.2 mg/mouse) was injected i.v. into female ICR mice which were anesthetized with Metofane (Pitman-Moore, NJ) and killed by cervical dislocation at 3 h post injection. The 3 hour time point was chosen because $t_{1/2}$ of liposome clearance are generally between < 1 and 7 h for liposomes composed of egg PC and cholesterol, and having an average diameter of approx. 200 nm [17]. ^{111}In radioactivity of each internal organ was counted in a gamma counter. Weight of mouse blood was assumed to be 7.3% of the body weight. Blood contamination in each organ was corrected [18].

TABLE I

Biodistribution of liposomes containing negatively charged DOPE derivatives

Liposomes composed of egg PC/cholesterol/DOPE derivative (10:5:1) were labelled with ^{111}In-DTPA-SA and i.v. injected into mice which were killed 3 h later. Data represent mean (S.D.), $n = 3$.

Lipids	^{111}In distribution (% injected dose)		
	blood	spleen	liver
DOPE	6.2 (2.1)	13.1 (1.9)	61.8 (4.4)
N-Malonyl DOPE	1.6 (0.4)	14.1 (1.9)	81.4 (1.9)
N-Succinyl DOPE	1.8 (0.2)	10.8 (5.0)	81.3 (2.2)
N-Glutaryl DOPE	31.1 (3.8)	9.2 (0.9)	43.8 (1.7)
N-Adipyl DOPE	28.6 (0.4)	9.0 (0.2)	51.4 (1.6)
N-Pimelyl DOPE	11.6 (3.1)	10.2 (1.6)	57.5 (5.7)
N-Suberyl DOPE	6.3 (0.7)	10.4 (1.1)	60.6 (1.9)
G_{M1}	51.3 (5.4)	6.8 (2.4)	28.8 (4.4)
None	8.1 (2.4)	11.4 (2.0)	68.4 (5.6)

Generally it has been observed that liposomes containing negatively charged phospholipids are more rapidly removed from circulation and localized in the RES cells of liver, spleen, and bone marrow than the neutral or positively charged liposomes [8–12]. Such a generalization was found to be the case for liposomes containing some of the DOPE derivatives. Incorporation of NMPE and NSPE into egg PC/cholesterol liposomes appeared to enhance the liposome uptake by RES cells while incorporation of DOPE itself did not (Table I). Liver uptake of liposomes containing NMPE and NSPE was about 81% of injected dose as compared to about 68% for liposomes containing no DOPE derivatives. Unexpectedly liposomes containing NGPE or NAPE were retained longer in the circulation with concomitant decrease in the RES uptake compared with egg PC/cholesterol liposomes. Incorporation of NPPE and NSBPE into liposomes had little effect on either liposome circulation or RES uptake. Therefore, the effectiveness of negatively charged DOPE derivatives, $HOOC(CH_2)_nCONH$-DOPE, in prolonging liposome circulation was dependent on the chain length of hydrocarbon between the amide and the terminal carboxyl group (Fig. 1). DOPE derivatives with $n = 1$ or 2 accelerated the clearance of liposomes from circulation while those with $n = 3$ or 4 delayed the clearance. Derivatives with a longer hydrocarbon chain than $n = 4$ appeared not to affect the liposome clearance in either way.

Gabizon and Papahadjopoulos [3] have divided various negatively charged lipids in two categories. A diacetylphosphate type of lipid has negatively charged groups which are exposed to the aqueous environment. The exposed negative charge promotes opsonization of liposomes via charge-mediated interaction with certain

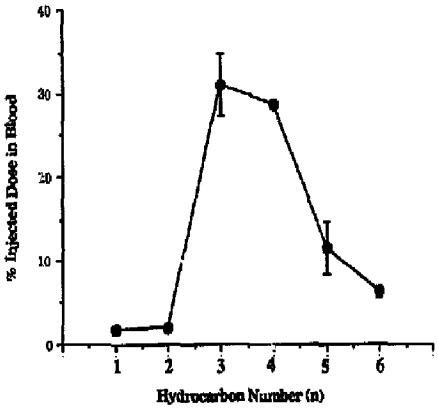

Fig. 1. Effect of DOPE derivatives on liposome concentration in the blood. Liposomes composed of egg PC/cholesterol/DOPE derivatives (10:5:1) were labeled with ^{111}In-DTPA-SA and injected into mice. Percent injected dose in blood was measured 3 h post injection and plotted against the hydrocarbon number (n) of the general formula, $HOOC(CH_2)_nCONH$-DOPE.

proteins in serum. The other type of lipid is some glycolipids such as G_{M1}, phosphatidylinositol (PI), or sulfatides, which have a negative charge shielded by surrounding bulky, neutral, hydrophilic groups. ^{13}C- and ^{1}H-NMR studies indicate that the G_{M1} structure is stabilized by an interaction between sialic acid and the galactosamine residue [19,20]. Other evidence on headgroup conformation of G_{M1} suggests that the carboxyl group of sialic acid and some hydroxyl groups of the galactose residue form an internal complex [21]. On the basis of these previous reports, it has been suggested that the shielded negative charge of G_{M1} may be responsible for the prolonged circulation of liposomes containing G_{M1} [2,3]. A similar hypothesis has been made for the activities of PI and sulfatides [3]. The hypothesis has accurately predicted the activity of prolonging liposome circulation time of poly(ethylene glycol)-phospholipid conjugates [5] which contain a negatively charged phosphate group shielded by a poly(ethylene glycol) chain. However, our observation is not consistent with the hypothesis, because negatively charged phospholipids with the exposed and unshielded carboxylic group such as NGPE and NAPE show considerable activity to prolong the circulation time of liposomes.

It has been suggested that she hydroxyl groups of G_{M1} and the poly(ethylene glycol) chains of the phospholipid conjugates confer a hydrophilic barrier to prevent opsonization [2,22]. In order to see whether there is any possibility to form a steric barrier on the liposome surface by the negatively charged DOPE derivatives, the effect of these derivatives on liposome agglutination was measured. Liposomes were prepared from egg PC/cholesterol/DOPE derivatives or G_{M1} (10:5:1, molar ratio) and 2.5 mol% *N*-biotinyl PE (Avanti Polar Lipid). 50 μl liposomes (1 mg lipid/ml) were mixed with 0.5 ml PBS in a microcuvette, and 5 μg streptavidin was added. Increase in turbidity was monitored as the optical density at 440 nm. We have previously shown that such measurement sensitively reveals the steric barrier activity of G_{M1} and the poly(ethylene glycol) derivatives of phospholipids [4,17]. Liposome agglutination mediated by streptavidin was not inhibited by the presence of NGPE and NAPE while it was significantly inhibited by G_{M1} (Fig. 2). Therefore, the possibility of a steric barrier presented by NGPE and NAPE can be excluded as an explanation for the action of NGPE and NAPE to prolong the liposome circulation time.

It is not simple to present a molecular model for the observation described here. For the derivatives with a short hydrocarbon chain, i.e., NMPE and NSPE, the position of the carboxylic group is close to the interfacial surface of liposome. Non-specific adsorption of opsonins responding to the surface negative charges may be responsible for the increased RES uptake of liposomes containing these two derivatives, much similar to the liposomes containing negatively charged phospholipids such as PS and PG. Chonn et al. [23] have reported that liposome adsorption of the activated complement component C3, a liposome opsonin [24,25], is significantly enhanced with the presence of negatively charged phospholipid. It is difficult to imagine that such nonspecific adsorption of the opsonin(s) would be completely inhibited when the length of

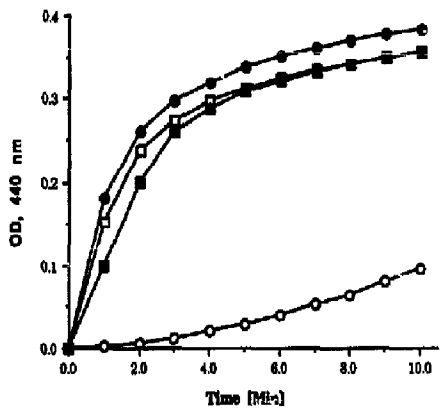

Fig. 2. Effect of NGPE and NAPE on the streptavidin-induced agglutination of liposome containing *N*-biotinyl-PE. The turbidity increase (optical density at 440 nm) was measured with time for liposomes containing none (●), G_{M1} (○), NGPE (■), and NAPE (□).

hydrocarbon chain increases by only one methylene unit. An alternative mechanism, despite speculative, would involve the adsorption of dysopsonin to the liposome surface, which might require that the terminal carboxylic group should be located a certain distance from the liposome surface. The activity of dysopsonin is to decrease the uptake of the liposomes by the RES [7]. DOPE derivatives with the longer hydrocarbon chain such as NPPE and NSBPE did not prolong the circulation time of liposomes. A simple explanation for this observation is that the terminal carboxylic group may be H-bonded with a nearby phosphate headgroup due to the flexibility of the longer hydrocarbon chain. These carboxyl groups would not be available for the dysopsonin recognition.

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