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Phosphatidylserine as a determinant of reticuloendothelial recognition of liposome models of the erythrocyte surface

(drug delivery/merocyanine 540/lipid packing/gangliosides)

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ABSTRACT **Liposomes formulated to resemble the outer leaflet of the erythrocyte membrane were found to substantially avoid recognition and clearance by the reticuloendothelial system. When these models of the erythrocyte surface were modified by the incorporation of >2 mol % of phosphatidylserine (PtdSer), their ability to remain in the circulation of mice was greatly reduced. To examine whether this altered behavior was the consequence of an alteration in bilayer organization induced by PtdSer, a method utilizing the fluorescent dye merocyanine 540 was used to assess the packing of external phospholipids. No significant difference in overall membrane lipid organization was detected between liposomes containing 2 or 3 mol % of PtdSer, at which dramatic differences in recognition and clearance occurred. These results exclude alterations in phospholipid packing as an indirect cause of increased clearance of PtdSer-containing liposomes and implicate PtdSer directly in recognition by the reticuloendothelial system.**

The practical application of liposomes as carriers for pharmacologically active substances *in vivo* has been frustrated by their rapid removal from the circulation (1–3). Because erythrocytes circulate for extended periods, their surface chemistry may guide the construction of liposomes that can avoid recognition by the reticuloendothelial system (RES) and thus persist in the circulation.

Both glycoproteins and glycolipids are presented on the erythrocyte surface. In mammals these molecules generally terminate in sialic acid residues. Beneath this exterior is the lipid bilayer with its asymmetric transbilayer distribution of phospholipids: phosphatidylcholine (PtdCho) and sphingomyelin are the major constituents of the exterior leaflet, whereas phosphatidylserine (PtdSer) and, to a lesser extent, phosphatidylethanolamine (PtdEtn) are restricted to the inner leaflet (4). The two leaflets of the erythrocyte membrane also differ in lipid packing: the exterior leaflet is more ordered (5, 6) and less fluid (7), and its lipids are more tightly packed (8) than the inner leaflet.

Large unilamellar liposomes containing PtdCho, sphingomyelin, and the ganglioside GM1 simulate several of these surface characteristics of erythrocytes and circulate for extended periods of time, substantially avoiding uptake by the RES (9). Besides providing a more efficacious drug delivery vehicle, these models of the erythrocyte surface provide a powerful means for assessing the importance of specific membrane properties in recognition and clearance by the RES. For example, when erythrocytes are treated with neuraminidase to remove sialic acid, they are rapidly removed from the circulation by uptake into the RES (10, 11). Similarly, when sialic acid is removed from the liposome models of the erythrocyte surface by neuraminidase, or by the omission of GM1, their affinity for the RES increases greatly (9). These results with erythrocytes and their models reinforce each other and imply that the presentation of exterior sialic acid contributes to nonrecognition by the RES.

Abolition of the asymmetric distribution of phospholipids in the erythrocyte membrane also results in enhanced recognition by cells of the RES (12, 13). Shuffling of external and internal leaflet lipids results in two alterations in the surface, which could provide a signal for recognition: the appearance of PtdSer on the exterior of the cell (14–16) and a looser packing of the phospholipids in the exterior leaflet (17)—perhaps, at least partially, as a consequence of introducing PtdSer into the exterior leaflet. Because these two signals are not normally dissociable in lipid-symmetric erythrocytes, liposomes offer an attractive alternative for assessing independently the potential role of each of these factors in RES recognition. In fact, altering the packing of phospholipids has already been shown to modify the circulatory ability of liposomes containing PtdCho and GM1 (9). We show here that the insertion of PtdSer into liposomes that model the erythrocyte surface results in their clearance by the RES without significant change in lipid packing and that clearance requires a threshold concentration of PtdSer. We also demonstrate that this effect is specific to the PtdSer headgroup in that it cannot be mimicked by other negatively charged phospholipids, such as phosphatidic acid or sulfoglycosylsphingolipids (formerly, sulfatides).

MATERIALS AND METHODS

Liposomes. GM1 gangliosides (Supelco, Bellefonte, PA) were dissolved in chloroform/methanol (2:1), and aliquots were added to appropriate mixtures of egg PtdCho, bovine brain sphingomyelin, phosphatidic acid, egg PtdEtn and PtdSer (Avanti Polar Lipids), and bovine brain sulfoglycosylsphingolipids (Sigma) in chloroform before evaporation of organic solvent. To radioactively label liposomes, ^{125}I-labeled tyraminylinulin (18) in 100 mM NaCl/0.1 mM EDTA/5 mM 2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid, pH 7.4, was added during liposome preparation at a concentration such that each mouse received 10^5–10^6 cpm of entrapped label. Large unilamellar liposomes prepared according to Szoka and Papahadjopoulos (19) and extruded through a 0.2-μm filter were consistent in size from sample to sample and averaged 0.16 ± 0.05 μm in diameter as determined by laser light scatter (Nicomp Instruments, Santa Barbara, CA). Multilamellar liposomes were prepared from dilauroyl or dipalmitoyl PtdCho (Sigma) as described (20).

***In Vivo* Clearance Assays.** Outbred female Institute for Cancer Research (ICR) mice (Animal Breeding Unit of the University of Alberta) were injected (three animals per

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Abbreviations: PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; RES, reticuloendothelial system; MC540, merocyanine 540.

group) in the tail vein with 0.5 mg of phospholipid in 0.2 ml of sterile saline (150 mM NaCl/20 mM sodium phosphate, pH 7.4). Control mice were injected with free ^{125}I-labeled tyraminylinulin, which is rapidly eliminated from the body by the kidneys (9, 18). Whole organs, carcass, or 100 μl of blood were obtained at 2 hr after injection, and radioactivity was counted in a Beckman 8000 γ counter; at this time recovery of injected radioactivity was 70–80% in all experiments and was not influenced by tissue distribution. The carcass generally contained <10% of the recovered radioactivity. With erythrocytes prelabeled with the lipid-soluble $^{111}In^{3+}$-labeled 8-hydroxyquinoline ($^{111}In^{3+}$-oxine) according to the technique of Hwang *et al.* (21), blood volume was determined to be 7.7% of body weight, and radioactivity in the tissues was corrected for the label present in blood.

Merocyanine 540 (MC540) Binding Assays. MC540 (Eastman) was diluted from a stock solution (1 mg/ml of water) to a concentration of 2 μg/ml in 0.5 ml of 100 mM NaCl/0.2 mM EDTA/20 mM Tris acetate, pH 8.1, in a microfluorescence cuvette. After determination of the emission spectrum (Spex Fluorolog 2 spectrofluorimeter) at an excitation wavelength of 565 nm, liposomes were added to the cuvette in successive aliquots to the amounts indicated in the figures, and emission spectra were again taken. From these spectra, fluorescence at 590 nm was determined and plotted as a function of phospholipid concentration (20). For titration of dimers, multilamellar liposomes of dilauroyl PtdCho were added to the MC540 solution until fluorescence was maximally depressed—usually at ≈80 nmol/ml. This mixture then served as the starting point for titrations done as just described. All titrations and measurements were made at room temperature.

RESULTS

Large unilamellar liposomes formulated from equimolar portions of PtdCho and sphingomyelin and containing ganglioside GM1 at a mole ratio of 0.14 with respect to either phospholipid were radioactively tagged with ^{125}I-labeled tyraminylinulin and injected into the circulation of mice. Two hours after injection the mice were sacrificed, and the amount of label in the liver, spleen, and blood was determined. As shown in Fig. 1, the liposomes were still found primarily in the circulation at this time. Addition of PtdSer to this mixture of lipids at a mole ratio of 0.14, or 2 mol %, of the other phospholipids (PtdCho plus sphingomyelin) had little effect on circulation of the resulting liposomes. However, addition of PtdSer to a level of 3 mol % or greater dramatically decreased levels of circulating liposomes. The liposomes lost from the blood were recovered largely in the liver, and a smaller portion was recovered in the spleen (Fig. 1).

The ability of liposomes to remain in circulation can be expressed as the blood/RES ratio, calculated by dividing the radioactivity remaining in the blood by the combined radioactivity found in the liver and spleen. Calculated blood/RES ratios at 2 hr after injection are shown in Table 1 for liposomes of several compositions. Values for liposomes composed of sphingomyelin/PtdCho/GM1 and containing 10 mol % of PtdSer were lower by a factor >150 than those for liposomes lacking PtdSer (experiments 8 and 3). The requirement for a threshold concentration of PtdSer for recognition by the RES was demonstrated by a comparison of the blood/RES ratio for liposomes containing 3 mol % of PtdSer, which was reduced by a factor of 25 (experiments 5 and 3), with that for liposomes containing 2 mol % of PtdSer, where no such dramatic effect was seen (experiments 4 and 3). Although GM1, in the absence of PtdSer, could prolong the circulatory survival of liposomes composed of sphingomyelin/PtdCho (experiments 3 and 1), in the presence of 10 mol % of PtdSer the blood/RES ratios were independent of the presence of GM1 (experiments 2 and 8), indicating that PtdSer could overcome the protective effect of GM1.

PtdEtn is another phospholipid found predominantly in the inner leaflet of erythrocytes. When asymmetry is lost, its level on the surface of the cell also increases. However, in contrast to PtdSer, when PtdEtn was included in sphingomyelin/PtdCho/GM1 liposomes at the 10 mol % level, the blood/RES ratio was only marginally different from controls (experiments 9 and 3).

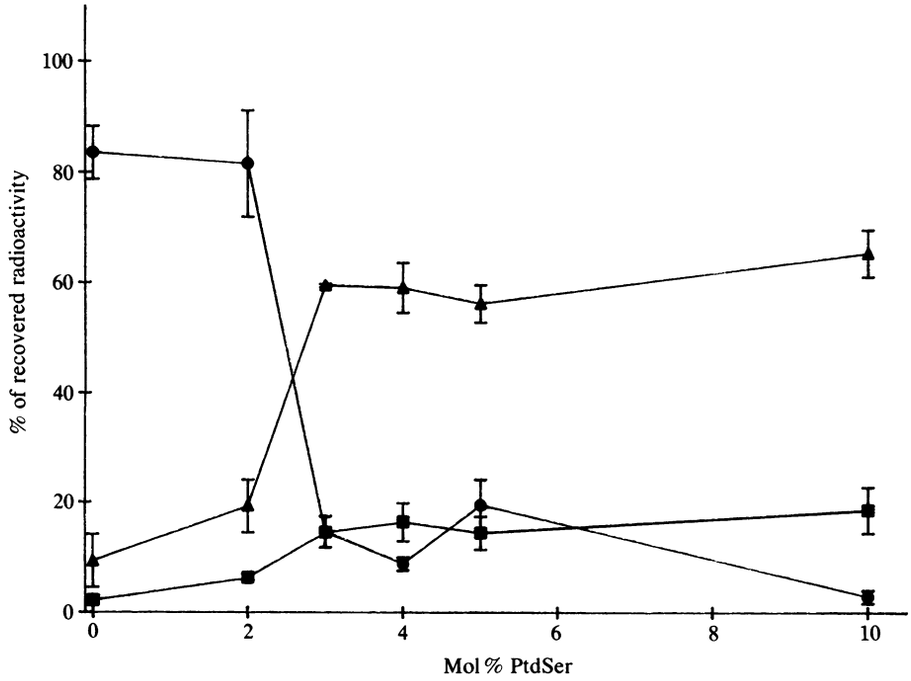

FIG. 1. Distribution of radioactivity in mouse tissues as a function of mol % PtdSer in liposomes. Percent of recovered radioactivity found in liver (▲), spleen (■), and blood (●) at 2 hr after injection.

Table 1. Effect of lipid composition on uptake of liposomes by the RES

Exp.	Lipid added	Mol ratio*	Blood/RES†
SM/PtdCho (1:1)			
1	None	—	1.4 ± 0.3
2	PtdSer	0.20 (10%)	0.02 ± 0.00
SM/PtdCho/GM1 (1:1:0.14)			
3	None	—	4.9 ± 2.0
4	PtdSer	0.04 (2%)	3.1 ± 0.7
5	PtdSer	0.06 (3%)	0.20 ± 0.04
6	PtdSer	0.08 (4%)	0.12 ± 0.01
7	PtdSer	0.10 (5%)	0.28 ± 0.07
8	PtdSer	0.20 (10%)	0.03 ± 0.07
9	PtdSer	0.20 (10%)	1.83 ± 0.61
10	Phosphatidic acid	0.20 (10%)	1.07 ± 0.63
11	Sulfoglycosyl-sphingolipids	0.20 (10%)	2.86 ± 1.36

SM, sphingomyelin.
*Numbers in parentheses are mol % of (sphingomyelin + PtdCho).
†Ratio of % injected radioactivity in blood/% injected radioactivity in liver plus spleen at 2 hr after injection; mean ± SD, $n = 3$.

To determine whether negative charge *per se* was the important factor in determining uptake, negatively charged sulfoglycosylsphingolipids (experiment 10) or phosphatidic acid (experiment 11) were incorporated into liposomes at 10 mol %. Little increase in uptake of these liposomes was seen as compared with liposomes lacking negative charge, implying that the PtdSer headgroup itself, rather than its charge, is required for recognition by the RES.

PtdSer derived from natural sources often contains a high level of unsaturated fatty acid side chains. Its inclusion in liposomes might therefore loosen the packing of the bilayer. Because a looser packing of phospholipids enhances clearance of liposomes (9), it was necessary to exclude the possibility that PtdSer alters recognition indirectly by altering bilayer organization. To assess the bilayer packing of liposomes, the fluorescent lipophilic probe MC540, which binds preferentially to bilayers in which the lipids are loosely packed (20), was used.

When MC540 enters the hydrophobic environment of a membrane bilayer, its fluorescence emission maxima is red-shifted and its fluorescence yield greatly increases. Affinity of liposomes for MC540 can thus be measured by monitoring the increase in fluorescence at the longer wavelength as a function of the addition of increasing quantities of liposomes to a constant amount of dye (20). This quantitative dye-partition assay is compromised, however, by a lag in the onset of increasing fluorescence upon liposome addition. This complication stems from the formation of nonfluorescent dye dimers in the membrane at high ratios of dye to lipid (20, 22). To obviate this problem, a modified assay was developed in which dye was presented in the form of membrane-bound, quenched dimers rather than as free monomer. Addition of test liposomes increases available bilayer binding sites, to an extent dependent on the affinity of the newly added membrane for the dye, with consequent monomer formation, dequenching, and increase in fluorescence. Kinetic analyses indicate that this equilibration is complete within ≈2 min (data not shown). As shown in Fig. 2*a*, application of this method to fluid-phase (dilauroyl PtdCho) or gel-phase (dipalmitoyl PtdCho) liposomes largely eliminated the lag in titration curves, whereas the large difference in the slope of the curves between these loosely and tightly packed bilayers (20) was retained.

When this assay was applied to standard PtdCho/sphingomyelin/GM1 liposomes, the addition of PtdSer did not increase the slope of the titration curve (Fig. 2*b*), indicating little change in the overall packing of the surface of the liposome. In particular, liposomes containing 2 mol % of PtdSer did not differ markedly in dye binding from those containing 3 mol %, even through these liposomes differed greatly in their recognition by the RES. Such results imply

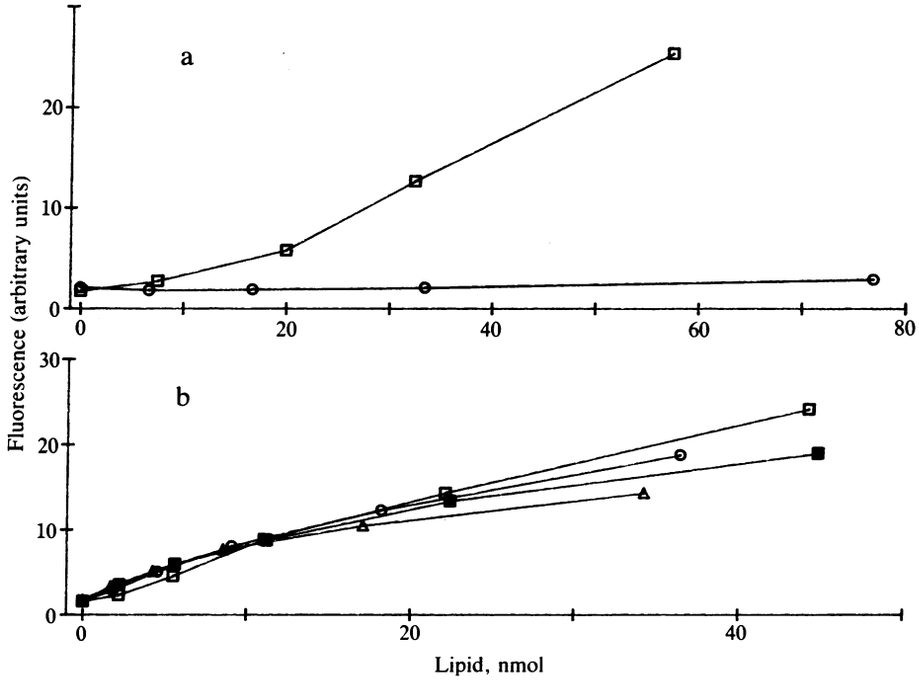

FIG. 2. Quenched dimer titrations of MC540 binding to liposomes. Liposomes were added to membrane-bound, quenched MC540, and the intensity of fluorescence emission at 590 nm was measured as a function of the amount of added lipid. (*a*) Liposomes made from fluid-phase dilauroyl PtdCho (□) or gel-phase dipalmitoyl PtdCho (○). (*b*) Liposomes made from sphingomyelin, PtdCho, and GM1 (1:1:0.14) containing no PtdSer (□), 2 mol % PtdSer (○), 3 mol % PtdSer (△), and 10 mol % PtdSer (■).

that the difference in circulation of these PtdSer-containing models of the erythrocyte surface does not result from a significant difference in lipid organization.

DISCUSSION

Confinement of certain phospholipids to the interior side of the erythrocyte plasma membrane is essential to maintenance of a noninteractive surface (17, 23, 24). When this asymmetric transbilayer distribution is lost, the phospholipids of the exterior leaflet become less tightly packed, as revealed by a several-fold increase in binding of MC540 (8), and interactions with cells of the RES are enhanced (12, 13). The strong correlation between the amount of dye bound by erythrocytes and their phagocytosis by macrophages led to the suggestion that a loosening of phospholipid packing can serve as a signal for recognition by the RES (13). This suggestion is supported by the finding that "rigidifying" lipids such as sphingomyelin (9) and cholesterol (9, 25) can enhance the circulation of liposomes formulated from PtdCho. Loose packing might enhance intercellular intereactions through increased exposure of the hydrophobic membrane core, increasing the hydrophobicity of the cell surface. In fact, hydrophobicity has long been known to regulate nonspecific recognition of bacteria by macrophages (26), and recognized symmetric erythrocytes do present a more hydrophobic surface than asymmetric erythrocytes (13).

Another consequence of loss of asymmetry is exposure of PtdSer on the exterior surface. Schroit and colleagues (14–16) propose that this single phospholipid species is itself a signal for recognition by the RES; they showed that introduction of an analog of PtdSer into the exterior leaflet of erythrocytes enhanced phagocytosis of the modified cells both *in vitro* and *in vivo*. However, insertion of the PtdSer analog could possibly increase spacing between external phospholipids enough to induce recognition by increasing hydrophobicity of the cell surface, just as the increased lipid spacing and increased binding of MC540 by lipid symmetric erythrocytes could simply accompany the only physiologically significant effect of asymmetry loss, exposure of PtdSer. Although these two mechanisms of recognition are not mutually exclusive, they are not dissociable in current whole erythrocyte systems. However, in a reconstituted system each variable can be manipulated independently of the other.

The composition of liposomes contributes markedly to their ability to escape recognition by the RES; liposomes that resemble the exterior leaflet of the erythrocyte in composition are particularly resistant to clearance (9). Surface sialic acid is required for enhanced circulation of liposomes made from PtdCho, itself also normally exposed on the exterior of erythrocytes. As shown here, addition of PtdEtn to the moderate levels normally found on the erythrocyte exterior has only minor effects on the ability of liposomes to circulate. Importantly, upon addition to egg PtdCho-based liposomes, the rigidifying lipids—sphingomyelin, distearoyl PtdCho, and cholesterol—all enhance circulation time (9).

The availability of these erythrocyte-surface models that exhibit a prolonged lifespan in the circulation allows us to test whether the introduction of PtdSer into the membranes can lead to RES recognition. Our results indicate that when levels of exposed PtdSer rise above ≈2 mol %, circulation time of the liposomes is dramatically reduced, with no correspondingly dramatic change in lipid packing, as assessed by MC540 binding. This effect was not a consequence of the net negative charge introduced at the bilayer surface by PtdSer, because addition of phosphatidic acid or sulfoglycosylsphingolipids at comparable or higher levels had only minor effect on the rate of removal. Thus, PtdSer itself appears to be a powerful signal for removal by the RES, suggesting that at least three different membrane properties—sialic acid, phospholipid packing, and PtdSer—can regulate membrane recognition *in vivo*. Preliminary studies of uptake of similar liposomes by bone-marrow macrophages suggest that the recognition mechanism is amenable to study *in vitro* (27).

It has been suggested that all blood cells, including platelets and leukocytes as well as erythrocytes, regulate their interactions with the RES by modulation of the lipids presented on their surface (17). Our findings may thus be relevant to the interactions involved in the immune and clotting responses. However, the practical aspects of these studies with models of the erythrocyte surface should not be overlooked. Although inclusion of PtdSer in liposomes may allow them to be targeted more efficiently to the RES, this goal is already attainable with liposomes of most compositions. The therapeutic advantages of these models of the erythrocyte surface, therefore, lie in regimens in which continued circulation of particulate carriers is desirable—for example, to allow time for other targeting mechanisms to function. For such applications these carriers offer promise.

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