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Gangliosides reduce leakage of aqueous-space markers from liposomes in the presence of human plasma

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We have studied the role of glycolipids in reducing leakage of aqueous-space markers from liposomes, composed primarily of egg phosphatidylcholine, in the presence of human plasma. Liposomes were either small unilamellar (SUV) or large unilamellar (LUV). Leakage of liposome contents as affected by the incorporation into the liposomal bilayer of mono-, di-, or trisialogangliosides (G_M, G_D, G_T) at different molar ratios in the presence or absence of cholesterol was examined. Leakage from liposomes decreased with increasing ganglioside sialic acid. Asialogangliosides had no effect on calcein leakage in the presence of plasma. The stabilizing effect of gangliosides and cholesterol was synergistic, and SUV containing 10 mol% G_T and 33 mol% cholesterol had a half-life for leakage of calcein in plasma at 37°C approaching 24 hours. LUV in the presence of plasma retained their contents longer than SUV, and gangliosides had an additional stabilizing effect. Phosphatidylserine and sulfatides were also capable of substituting for gangliosides in stabilizing liposomes to plasma-induced leakage. It appears that gangliosides stabilize liposomes in plasma at least in part through their ability to impart surface negative charge.

Introduction

The tendency of liposomes to become destabilized in the presence of serum has been described by several authors [1–4]. This tendency limits their utility as drug carriers in vivo. The stability of liposomes in plasma can be improved by incorporation of cholesterol in the liposomal bilayer [2–5], by manipulating liposome phospholipid composition [6], and by photopolymerizing phospholipid vesicles [7].

The mechanism of destabilization of liposomes in the presence of serum is thought to be due to transfer of liposomal phospholipid to, and exchange of phospholipid with, high-density lipoproteins [5,6,8,9]. However, complement-dependent immune damage to liposomes, mediated by naturally occurring anti-liposome antibodies, may also be involved [10,11]. Finkelstein and Weissmann [10] have reported a reduction in plasma-induced leakage from multilamellar liposomes after treat-

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Abbreviations: SUV, small unilamellar liposomes; LUV, large unilamellar liposomes; egg PC, egg phosphatidylcholine; PS, bovine brain phosphatidylserine; G_T, trisialoganglioside (predominantly G_{T1b}, $IV^3NeuAc,II^3(NeuAc)_2$-$GgOse_4Cer$); G_D, disialoganglioside (predominantly G_{D1a}, IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$); G_M, monosialoganglioside (predominantly G_{M1}, II^3NeuAc-$GgOse_4Cer$); DPPC, dipalmitoylphosphatidylcholine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

ment with decomplemented serum. Heating of serum, however, in addition to destroying complement, also inactivates a protein which appears to act in conjunction with HDL in transferring or exchanging phospholipids from liposomes [12,13]. In addition to plasma lipoproteins, a variety of other plasma proteins are absorbed to liposome surfaces and may be involved in their destabilization and removal from circulation [14,15].

We have examined the effect of incorporation into liposomal bilayers of mono-, di- and trisialogangliosides on plasma-induced leakage of liposome contents. These experiments were designed to test the possibility that surface sialic acid will prevent or moderate the interaction of plasma proteins with liposomal surfaces, and thereby reduce leakage of liposome contents. Small unilamellar liposomes (SUV) were used in the majority of these studies because we were interested in eventual in vivo applications and SUV have longer circulation times then larger liposomes [16].

Materials and Methods

Egg phosphatidylcholine (egg PC), bovine phosphatidylserine (PS), and dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti Biochemicals (Birmingham, AL); cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized twice from methanol. Calcein was purchased from Hach Co. (Ames, IA) and was purified on a Sephadex LH – 20 column [17]. Sulfatides (bovine) were obtained from Supelco, Inc. (Bellefonte, PA). Outdated human plasma (citrate/phosphate/dextrose) was donated by the Irwin Memorial Blood Bank (San Francisco, CA). [^{14}C]Inulin (4 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

Gangliosides

Gangliosides were prepared from human brain by the method of Momoi et al. [18]. The brain was homogenized in 1 : 2 (v/v) chloroform-methanol solution overnight. The extracted brain was filtered through a Whatman No. 1 filter and the filtrate was applied to a DEAE-Sephadex A-25 column. Gangliosides and other acidic lipids were eluted with 0.2 M sodium acetate in methanol, saponified with 0.1 M sodium hydroxide, and dialyzed against water at 4°C overnight. The crude ganglioside preparation was lyophilized and resuspended in 95 : 5 (v/v) chloroform-methanol. The suspension was applied to a column of Iatrobeads (Iatron Corp., Tokyo, Japan) and eluted with a stepwise gradient of chloroform/methanol. Fractions were assayed for ganglioside content by thin-layer chromatography on HPTLC plates, using resorcinol spray to visualize sialic acid [19]. The ganglioside-containing fractions were pooled and separated into sialic acid classes by chromatography on DEAE-Sephadex A-25, using a methanol-ammonium-acetate gradient for elution [18]. Each sialic acid class (G_M, G_D and G_T) was concentrated by rotary evaporation, dialyzed against water, and lyophilized.

Asialoganglioside was prepared from purified trisialoganglioside (G_T) by formic acid hydrolysis [20]. 20 mg of G_T were hydrolyzed in 2 ml 0.1 M formic acid for 2 h at 100°C. After cooling, the ganglioside was partitioned in chloroform/methanol/water (8 : 4 : 3, v/v). The lower phase was washed once with the upper phase to fully remove sialic acid, and was evaporated to dryness under vacuum. The yield of asialogangliosides was approximately 50% as estimated by neutral sugar analysis [21]. Ganglioside concentrations in liposomes were determined by the resorcinol method [19].

Liposomes

Liposomes were prepared according to the following procedure. Phospholipids and cholesterol in the desired molar ratios were evaporated to dryness in a rotary evaporator. Dried lipid was resuspended in a 1 : 1 chloroform-methanol solution that contained dissolved ganglioside in the desired amount, and the mixture was dried down again in the rotary evaporator. Small unilamellar vesicles (SUV) were formed by hydrating the lipid in 50 mM calcein solution (220 mosM) at pH 7.4 and sonicating to clarity under argon in a bath-type sonicator (Laboratory Supplies, Inc., Hicksville, NY) in an enclosed glass container at 20°C for 0.5 to 1 h. Liposomes containing entrapped calcein were separated from free calcein by passage over Sephadex G-75 in buffer containing 100 mM NaCl, 0.1 mM EDTA, 5 mM Tes, pH 7.4 (buffer). Reverse-phase evaporation vesicles containing en-

trapped 50 mM calcein were prepared according to the technique of Szoka and Papahadjopoulos [22] and were extruded through 0.1 μm polycarbonate filters [23]. These vesicles are primarily unilamellar [24,25]. Half-lives for calcein leakage in the presence or absence of human plasma was measured in an SLM 4000 spectrofluorometer according to the technique of Allen and Cleland [3]. Briefly, 20 μl of liposomes containing 0.1 μmol of lipid was pipetted into 980 μl of buffer or plasma at the appropriate temperature, and fluorescence increase was monitored at 0.5 to 1 h intervals at excitation and emission wavelengths of 490 nm and 520 nm, respectively. Low liposome concentrations were chosen in order to approximate the ratio of liposomes to plasma components which would be expected if liposomes were injected in vivo. 100% leakage was determined by lysing the samples with 50 μl of 10% Triton X-100. We also lysed samples with 10% Zwittergen or 1.0% deoxycholate and found very similar maximum fluorescence, although the rate of lysis was slower. Addition of greater amounts of Triton X-100 did not substantially decrease the maximum fluorescence. The kinetics of leakage has been shown previously to be linear on a semi-logarithmic plot [3] and were also found to be linear for ganglioside-containing liposomes.

The reproducibility of half-life measurements was ascertained by measuring $T_{1/2}$ for egg PC in the presence or absence of 10 mol% G_T for four or more different samples in plasma and buffer. The standard deviation was approx. 30% of the mean. Because of a shortage of gangliosides only one sample was made for some of our measurements. Repeat measurements on the same sample were very similar to each other.

Results and Discussion

The half-time for leakage of calcein from egg PC liposomes at 37°C in buffer or in plasma was measured for SUV containing G_M, G_D or G_T (Table I). Gangliosides decreased leakage of aqueous solute from liposomes in the presence of buffer or plasma, and leakage in plasma decreased with increasing amounts of sialic acid associated with the gangliosides.

The effect of increasing G_T concentration on half-time of calcein leakage from SUV was assessed next (Table I). Liposomes containing 20 mol% G_T were more leaky in the presence of buffer than liposomes containing lower mol% of G_T. This may be attributed to the detergent-like effect of gangliosides that becomes apparent around 20 mol% [26], however, this effect was not apparent in plasma where the liposomes are already much leakier. As G_T concentration increased from 5 to 20 mol% in egg PC liposomes, leakage of solute in the presence of plasma decreased. A concentration of 30 mol% sialic acid (10 mol% G_T) was chosen for subsequent experiments.

TABLE I

EFFECT OF INCORPORATION OF GANGLIOSIDES OR CHOLESTEROL ON LEAKAGE OF 50 mM CALCEIN FROM EGG PC SUV AT 37°C IN BUFFER OR PLASMA

Number in brackets indicate numbers of samples tested. CH, cholesterol.

	$T_{1/2}$ (h)		Encapsulated volume (liter/mol)
	buffer	plasma	
Egg PC	10.7 ± 3.0 (6)	0.4 ± 0.1 (6)	0.5, 0.4
Egg PC/G_M, 10:1	22.5, 20.2	0.8, 0.7	
Egg PC/G_D, 10:1	43.0, 22.6	1.1, 1.3	
Egg PC/G_T, 10:0.5	22.7	1.7	
Egg PC/G_T, 10:1	38.7 ± 10.2 (5)	2.1 ± 0.7 (5)	0.7, 0.6
Egg PC/G_T, 10:1.5	36	3.0	
Egg PC/G_T, 10:2	18.8	3.4	
Egg PC/CH, 2:1	18.1 ± 6.3 (5)	4.2 ± 1.2 (5)	0.7, 0.7
Egg PC/CH/G_T, 10:5:1	53.4 ± 8.5 (3)	18.8, 24.3	2.3, 2.1

Trisialoganglioside also had a stabilizing effect on SUV composed of DPPC both above and below the phase transition in buffer and in plasma, but SUV (DPPC) in the presence or absence of gangliosides were more leaky than SUV (egg PC) (results not shown). This instability of DPPC SUV in plasma has also been described by Senior and Gregoriadis [27] and is related to the phase transition of DPPC SUV which is reported to be 36.9°C [28].

The effect of cholesterol on calcein leakage in the presence or absence of 10 mol% G_T is also shown in Table I. Cholesterol had a small stabilizing effect on SUV (egg PC) in buffer in the presence or absence of 10 mol% G_T. A more marked stabilizing effect of cholesterol was observed in the presence of plasma at 37°C. 10 mol% G_T decreased leakage from liposomes 4-fold in the absence of cholesterol, and cholesterol decreased leakage 7-fold in the absence of G_T. When both were present, there was a 36-fold decrease in leakage of solute from liposomes in plasma. The effect of cholesterol and G_T appears to be synergistic in the presence of plasma, but this effect may be related in part to liposome size (see below). The state of aggregation of gangliosides (or their conformation) in the lipid bilayer may be altered by the presence of cholesterol such that membrane stability is enhanced.

In order to determine if sialic acid had a critical role in mediating the stabilizing effect of gangliosides on plasma-induced leakage of liposomal contents, we prepared asialoganglioside from G_T and measured $T_{1/2}$ for leakage for SUV (egg/PC cholesterol, 2 : 1) in the absence of ganglioside and in the presence of 10 mol% G_T or 10 mol% asialoganglioside. Half-times for leakage of calcein at 37°C in buffer and in plasma for liposomes containing 10 mol% asialoganglioside were identical to half-times for liposomes containing no gangliosides (results not shown). The stabilizing effect of gangliosides is therefore dependent on the presence of sialic acid and is not a property of the ceramide-oligosaccharide moieties of the molecules.

The interpretation of these results may plicated to some extent by the state of aggregation of the gangliosides in the bilayer. Recent studies provide evidence that G_{M1}, and other sialoglycolipids are monomolecularly dispersed in liquid-crystalline phosphatidylcholine bilayers [29–31]. However, neutral glycolipids like asialo G_{M1} are reported to be organized into domains in the absence of cholesterol [29,30]. Their organization in the presence of cholesterol is unknown. Galactosylceramide has been reported to be clustered in DPPC liposomes, in the absence of cholesterol, and randomly dispersed in its presence [32]. The state of organization of charged and neutral glycolipids in bilayers, the effect of cholesterol, phase-transition, divalent cations and other parameters on this organization, and the effect of glycolipid organization on other bilayer properties remains to be resolved.

We determined $T_{1/2}$ for leakage in buffer at 25°C of [^{14}C]sucrose and [^{14}C]inulin from SUV (egg PC) in the presence and absence of G_T, in order to ensure that the negative charge of calcein was not responsible for the observed effects. The $T_{1/2}$ for leakage of sucrose was 98 ± 3 h ($n = 3$) in the absence of gangliosides and greater than 800 h ($n = 2$) in the presence of 10 mol% G_T (as compared to 54 h for calcein at 25°C). No detectable leakage of [^{14}C]inulin was observed over a 27-h period. It appears, therefore, that the presence of ganglioside has a similar effect on the leakage of an uncharged solute (sucrose) and a negatively charged solute (calcein).

Trapped volumes of SUV containing both cholesterol and gangliosides (egg PC/cholesterol/G_T, 10 : 5 : 1) were consistently 4- to 5-fold larger than trapped volumes of SUV containing either compound alone and averaged 2.2 ± 0.1 liter/mol (Table I). Both egg PC/G_T (10 : 1) SUV and egg PC/cholesterol (2 : 1) SUV had slightly larger trapped volumes than egg PC SUV. The effect of cholesterol on increasing SUV size has been reported previously [33]. It has been reported [9] that larger liposomes are less susceptible to the action of plasma than smaller liposomes, and therefore the apparent larger size of liposomes (egg PC/cholesterol/G_T) may contribute to their increased stability in plasma. In order to examine this possibility further, we compared half-times for leakage of calcein at 37°C in buffer or plasma for SUV (egg PC), 0.1 μm LUV and 0.2 μm LUV in the presence and absence of G_T (Table II). The trapped volume of the LUV is also given in Table

II. The increased size of the SUV does not fully explain their incresaed stability as egg PC LUV (0.2 μm), were less resistant to plasma components than 0.1 μm LUV, although they had increased stability in buffer. Furthermore, the stabilizing effect of G_T was apparent also in larger liposomes (LUV), indicating that the stabilization is not strictly related to size changes.

Because gangliosides, which carry negatively charged sialic acid, have a stabilizing effect on liposomes, we have explored the effect of incorporation of phosphatidylserine (PS) and sulfatides, both of which carry a net negative charge, on leakage of calcein from liposomes (Table III). Leakage rates in plasma of calcein from liposomes containing 10 mol% PS or 10 mol% sulfatides were similar to those from liposomes containing 10 mol% G_M (same total negative charge), and leakage rates from liposomes containing 30 mol% PS were similar to liposomes containing 10 mol% G_T (same total negative charge). Very large amounts of negative charge appear to cause increased leakage in the presence of plasma and 30% to 50% negative charge appears to be optimum (Table III). The effects of PS and sulfatides on calcein leakage from liposomes in buffer were not as apparent. The stabilizing effect of ganglioside appears to be related, at least in part, to the negative charge contained in the sialic acid moiety.

Surface negative charge may inhibit the binding of high-density lipoproteins to liposome surfaces and thereby slow down the lipoprotein-mediated disintegration of phospholipid vesicles. Sialoglycolipids may also have a more specific effect in preventing the binding of immune proteins to liposomal surfaces. Okada et al. [34] have reported that sialoglycolipids on liposome membranes containing trinitrophenylaminocaproyldipalmitoyl phosphatidylethanolamine restrict activation of the alternative complement pathway (ACP), and that removal of the terminal sialic acid from the glycolipids abolishes this restricting capacity. Insertion of negative charge into liposome membranes (dicetyl phosphate or dipalmitoylphosphatidic acid) had no effect on ACP activation. In circumstances where complement activation is involved in destabilization of liposomal membrane, gangliosides may have a protective effect not shared by other negatively charged substances.

Gangliosides are ubiquitous components of eukaryotic plasma membranes, with multiple functions including growth modulation and receptor functions [35]. These experiments show, for the first time, that gangliosides may also modulate the stability of plasma membrane in the presence of serum. Experiments to examine the in vivo significance of these observations are currently underway, as it is possible that sialoglycolipids will affect liposome clearance in a difference manner than other negatively charged lipids.

TABLE II

EFFECT OF LIPOSOME SIZE ON THE HALF-TIMES FOR LEAKAGE OF CALCEIN FROM EGG PC VESICLES IN THE PRESENCE OR ABSENCE OF 10 mol% G_1 AT 37°C

	$T_{1/2}$ (h)		Encapsulated volume (liter/mol)
	buffer	plasma	
Egg PC LUV 0.1 μm	35.3	8.7, 5.2	2.0
Egg PC/G_T, 10:1 LUV 0.1 μm	110.1	15.0, 17.2	3.3
Egg PC LUV 0.2 μm	52.1	5.4, 4.0	2.8
Egg PC/G_T, 10:1 LUV 0.2 μm	96.8	8.5, 5.0	4.7

TABLE III

EFFECT OF NEGATIVE CHARGE ON THE HALF-TIME FOR LEAKAGE OF CALCEIN FROM EGG PC SUV IN BUFFER OR PLASMA AT 37°C

	% negative charge	$T_{1/2}$ (h)	
		buffer	plasma
Egg PC	0	10.7 ± 3.0 (6)	0.4 ± 0.1 (6)
Egg PC/G_M, 10:1	10	22.5, 20.2	0.8, 0.7
Egg PC/PS, 10:1	10	19.9, 19.9	0.7, 0.7
Egg PC/SO_4, 10:1	10	10.6	0.6
Egg PC/G_T, 10:1	30	38.7 ± 10.2 (5)	2.1 ± 0.7 (5)
Egg PC/PS, 10:3	30	14.5	2.2
Egg PC/PS, 1:1	50	19.3, 13.9	1.7, 2.6
Egg PC/PS/G_1, 10:10:1	80	30.7, 54.7	1.0, 0.9

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