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pH-sensitive, plasma-stable liposomes with relatively prolonged residence in circulation

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Acid-sensitive liposomes composed of unsaturated phosphatidylethanolamine (PE) are efficient vehicles for cytoplasmic delivery of the target cells. We have recently shown that liposomes composed of dioleoyl-PE (DOPE) and dipalmitoyl-succinylglycerol (DPSG) retain the acid-sensitivity after exposure to human plasma. In the present work, we have extended these observations to investigate the role of ganglioside GM_1 on the blood residence time of these liposomes. Small ($d \approx 100$ nm) unilamellar liposomes composed of DOPE and DPSG (4:1, molar ratio) became progressively less acid-sensitive when increasing amounts of GM_1 were included in the lipid composition. However, partial sensitivity to acid (40–50% release of entrapped contents at pH 4) could be retained up to 5% GM_1, even for liposomes which had been exposed to human plasma. Inclusion of GM_1 in the lipid composition only slightly increased the release of entrapped contents in the presence of human plasma. The biodistribution of i.v. injected GM_1-containing liposomes was studied by following the entrapped ^{125}I-labeled tyraminylinulin marker in Balb/c mice. Inclusion of up to 5% GM_1 showed a transient increase in the blood level and a concomitant decrease of liver and spleen uptake of liposomes. Thus, these liposomes are pH-sensitive, plasma-stable and show a relatively prolonged residence time in circulation. They are potentially significant drug carriers in vivo.

Introduction

Liposomes, or phospholipid vesicles, have been extensively studied for their potential use as drug carriers (for a recent book, see Ref. 1). pH-sensitive liposomes composed of unsaturated phosphatidylethanolamine (PE) and weakly acidic amphiphiles are effective in the cytoplasmic delivery of the entrapped contents to the target cells [2–7]. These liposomes become destabilized and fusion-competent when they encounter the acidic environment in the cellular endosomes and lysosomes, resulting in the release of the entrapped contents into the cellular cytoplasm [2–4]. Antitumor drugs [4], toxin [7] and DNA [6] have been efficiently delivered to the target cells using these liposomes.

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPSG, 1,2-dipalmitoyl-*sn*-3-succinylglycerol; PBS, phosphate-buffered saline; DOC, deoxycholic acid; PE, phosphatidylethanolamine; RES, reticuloendothelial system; OA, oleic acid.

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Although the pH-sensitive liposomes show great promise as a drug carrier, their poor in vivo stability has been problematic for further developments. We have previously shown that large unilamellar, pH-sensitive liposomes composed of DOPE/OA (2:1) are not stable in serum or plasma, primarily due to the rapid extraction of oleic acid from the liposome membrane by albumin. Small ($d \leqq 200$ nm) unilamellar liposomes of the same composition, on the other hand, show a striking stability in serum or plasma [8,9]. This is due to the fact that insertion of serum apolipoproteins into the liposome membranes of high curvature, i.e., the membranes of small liposomes, can give rise to a remarkable stability of the liposome even after the extraction of oleic acid by albumin [8,9]. Unfortunately, the serum-stabilized small liposomes are no longer pH-sensitive; no release of the entrapped contents can be observed as low as pH = 3 [9]. Therefore, these liposomes are unlikely to be useful for he cytoplasmic delivery in the target cells.

Recently, we [10] and others [11] have shown that small unilamellar liposomes composed of DOPE and a double-chain amphiphile, dipalmitoylsuccinylglycerol (DPSG), are pH-sensitive and relatively serum/

plasma-stable. Furthermore, these liposomes remain partially acid-sensitive after exposure to serum [10]. It has been hypothesized that the serum stability of these liposomes arises from the resistance of DPSG to albumin extraction, although no definitive evidence is given [10].

Another important development in the liposome field is the search for liposome compositions which allow the liposomes to stay in circulation for a relatively long period of time [12–14]. These liposomes have been called 'stealth' * because of their relatively low uptake by the reticuloendothelial system (RES) in liver and spleen [13]. One of the important ingredients for 'stealth' liposomes is the ganglioside GM_1 [12–14]. Inclusion of up to 6.25% GM_1 significantly increases the circulation time of liposomes and reduces the RES uptake such that the RES/blood ratio of the 'stealth' liposomes is 8.5-fold smaller than that of the ordinary liposomes composed of PC and cholesterol [12]. Furthermore, the uptake of the 'stealth' liposomes by solid tumor is significantly enhanced, suggesting that the prolonged circulation time can lead to a greater penetration of liposomes to the vesculatures of the tumor [12].

The therapeutic potential of liposomes as drug-delivery vehicles depends on the efficient delivery of liposome-entrapped compounds to cells. A number of studies [2–7] have demonstrated in culture cells that pH-sensitive liposomes show a high efficiency of delivery. However, these liposomes have not been successfully used in animals due to the poor stability of liposomes in buffer [8] and in serum [16], the loss of pH sensitivity after exposure to serum [8] and the rapid uptake by RES system [9]. In the present study, we have attempted to overcome these problems by optimizing the lipid composition of the liposomes. Our goal is to prepare liposomes which are acid-sensitive, plasma-stable and relatively 'stealth' for their potential use as drug carriers.

Materials and Methods

Materials

DOPE, DOPC and DPSG were purchased from Avanti Polar Lipids. GM_1 was obtained from Calbiochem. Calcein was purchased from Sigma Chemical Co. ^{125}I-labeled tyraminylinulin was prepared according to Sommerman et al. [15].

Liposome preparation

Phospholipid and DPSG (molar ratio 4 : 1) with different amounts of GM_1 were mixed and evaporated free of $CHCl_3$ with N_2 gas. The dried lipid films were vacuum desiccated and suspended in either 50 mM calcein in PBS (pH 8.0) or ^{125}I-tyraminylinulin (2 mg/ml in PBS, pH 8.0). The samples were then incubated 5 h at room temperature. The samples were sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) and the pH was maintained at 8.0 during sonication. Liposomes were left at room temperature overnight to facilitate the annealing process. Free calcein or inulin was separated from liposomes by passage over a Bio-Gel A-0.5m column equilibrated with PBS (pH 7.6) which was made isotonic to calcein solution by addition 10 × PBS. The final osmolality of these solutions was equal to 320 mosmol/kg, the osmolality of mouse plasma. The size of liposomes was measured by a dynamic laser light scattering using a Coulter NS4D instrument. To monitor the lipid concentration after chromatography, a trace amount of hexadecyl [^{3}H]cholestanyl ether (0.8 μCi/mmol lipid) was included in the samples prior to $CHCl_3$ evaporation. Lipid compositions are expressed as mol% or molar ratios.

Liposome stability assay

The stability of liposomes was monitored as previously described [16]. Percent leakage was calculated using the equation:

$$\% \text{ Release} = \frac{(F_t/F) \cdot F_x - F_0}{F_t - F_0} \quad (1)$$

where F_0 is the fluorescence intensity of the liposomes in PBS at room temperature at time zero. F_t is the total fluorescence intensity of liposomes in PBS after addition of DOC. F_x is the fluorescence intensity of the liposomes incubated in plasma at 37 °C at time x. F is the total fluorescence intensity of the liposomes in plasma after addition of DOC. F_t/F is a correction term which is used to minimize the pipetting and other systematic errors in the measurements. The value of F_t/F ranged from 0.95 to 1.05.

pH-sensitivity assay

10 μl of liposomes containing about 3 nmol lipid were added to a cuvette containing 1.99 ml of PBS at the desired pH, incubated for 5 min, and then adjusted to pH 8.0 by adding appropriate amounts of NaOH. Eqn. 1 was used to calculate % release. F_0 was the fluorescence intensity at pH 8.0 and F_x was the fluorescence intensity at pH 8.0 after incubation at different pH. F_t and F were the total fluorescence intensities of liposomes incubated at pH 8.0 and desired pH after lysis of liposomes with DOC.

Treatment of liposomes with plasma

Liposome suspensions containing 1 μmol lipid (in 100 μl) were incubated with an equal volume of human plasma for 2 h at 37 °C. Excess plasma proteins were separated from liposomes by chromatography on a Bio-Gel A-1.5m column.

* 'Stealth' is a registered trademark of Liposome Technology, Inc.

In vivo biodistribution

150 μl ^{125}I-tyraminylinulin-containing liposomes (1 μmol lipid, $(1-5)\cdot 10^6$ cpm) were administered intravenously by tail vein injection into Balb/c mice (6–8 weeks old, male). At the desired time, mice were anaesthesized with diethyl ether and bled by eye puncture. Blood was collected and weighed. The mice were killed by cervical dislocation. Organs were collected and counted for ^{125}I-counts in a gamma counter. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7.3% of the total body weight [17]. The counts in liver and spleen were regarded as the counts in RES [12].

Results

We have used a basic lipid composition of DOPE : DPSG (4 : 1) throughout the study. This composition gives rise to liposomes which are acid-sensitive and relatively plasma-stable [10]. Small unilamellar liposomes of average diameter ($d \leqq 120$ nm) prepared by sonication are used because it has been shown that the circulation time of small liposomes is generally longer than that of larger liposomes [18,19].

Effect of GM_1 on pH sensitivity of liposomes

Increasing amounts of GM_1 in DOPE/DPSG (4 : 1) liposomes have decreased the sensitivity of liposomes to acid treatments. This is shown by the release of the entrapped calcein from liposomes at different acidic pH (Fig. 1). As can be seen in Fig. 1A, release of calcein at pH 4–6 was progressively reduced when increasing amounts of GM_1 were included in the lipid composition. Only about 40% release of calcein was observed at pH 4 with the liposomes containing 5% GM_1, as compared with 100% release from liposomes containing no GM_1 at the same pH. Also shown in Fig. 1B is the pH-sensitivity of the same liposomes which had been treated with 50% normal human plasma and reisolated by chromatography. The release of calcein at acidic pH was reduced after the plasma treatment for liposomes containing 0 to 2.5% GM_1. However, the acid sensitivity of liposomes containing 5% GM_1 remained approximately the same after the plasma treatment. In fact, the calcein release curves for all plasma-treated liposomes were very similar to each other, indicating that a partial acid-sensitivity of liposome can be preserved in plasma regardless of the GM_1 content (up to 5%) of the liposome. Higher amounts of GM_1 (7.5 and 9%) further reduced the acid sensitivity of the liposomes, no matter whether the liposomes were treated with plasma or not (Fig. 2). Thus, the effect of plasma treatment on the acid sensitivity of liposomes diminishes as the GM_1 concentration increases. At high concentrations of GM_1 ($\geqq 5\%$), the acid sensitivity of liposomes was independent of the plasma treatment (Fig. 2). The lack of acid sensitivity of liposomes composed of DOPC/DPSG (4 : 1) is also shown in Fig. 1A, indicating the H^+-induced destabilization of liposome membrane is a characteristic feature of the PE containing liposomes.

Dissociation of DPSG from liposomes upon plasma treatment

[^{14}C]DPSG was used to study the dissociation of DPSG from liposomes during the plasma treatment. Liposomes containing [^{14}C]DPSG were separated from the plasma components by gel filtration using a Bio-Gel A-1.5m column. Liposomes were eluted in the void volume fractions as indicated by the quenched calcein fluorescence in these fractions. The plasma components including lipoproteins and albumin were eluted in the included volume fractions. Table I shows the distribution of [^{14}C]DPSG in the column fractions from liposomes with and without 5% GM_1. The results clearly indicate that DPSG was partially ($\approx 40\%$) transferred

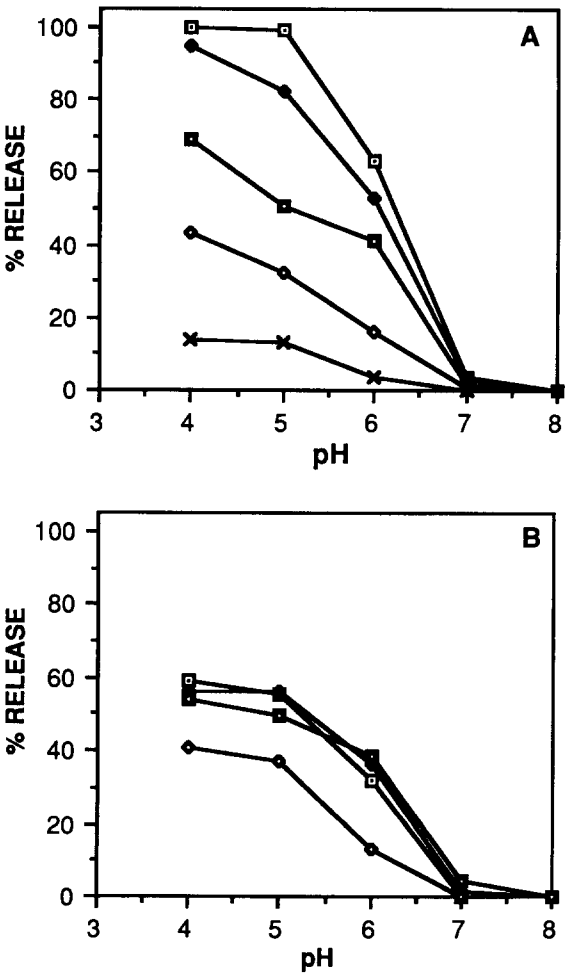

Fig. 1. Effect of GM_1 on pH sensitivity of liposomes. (A) PBS-treated and (B) plasma-treated liposomes composed of DOPE and DPSG. ▫, without GM_1; ◆, with 1% GM_1; ■, with 2.5% GM_1 and ◇, with 5% GM_1. ×, pH-insensitive liposomes composed of DOPC and DPSG (4 : 1).

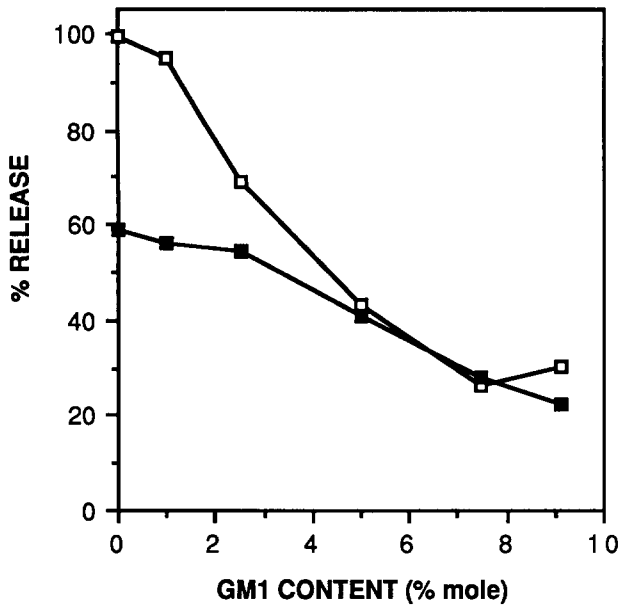

Fig. 2. Percent calcein release of liposomes composed of DOPE, DPSG with different amount of GM_1 at pH 4.0. □, PBS-treated; ■, plasma-treated.

to plasma components during the incubation, and that the transfer was not influenced by the inclusion of GM_1 in the liposome membrane.

Effect of GM_1 on th stability of liposomes in plasma

Allen et al. [20] have reported that GM_1 improves the stability of liposomes in serum. The liposomes used in that study were mainly composed of PC. It is thus important to examine the effect of GM_1 on the plasma stability of PE-containing liposomes. The release of the entrapped calcein from liposomes was measured during the incubation with 90% human plasma at 37°C (Fig. 3). Both DOPC- and DOPE-containing liposomes were studied. Calcein release from liposomes composed of DOPC/DPSG (4 : 1) was significantly reduced when 2.5 and 5% GM_1 were included in the lipid composition

TABLE I

Dissociation of DPSG from liposomes upon plasma treatment

[^{14}C]DPSG ($2 \cdot 10^5$ cpm/mg) was included in the liposome membrane. Liposomes were incubated with 50% normal human plasma for 2 h at 37°C before fractionation on a Bio-Gel A-1.5m column. Liposomes were eluted in the excluded volume, and lipoproteins and other plasma components were eluted in the included volume.

Lipid composition	Treatment	% [^{14}C]DPSG in	
		excluded vol.	included vol.
DOPE/DPSG (4:1)	PBS	100	0
DOPE/DPSG (4:1)	plasma	62.5	37.5
DOPE/DPSG/GM_1 (76:19:5)	plasma	60.7	39.3

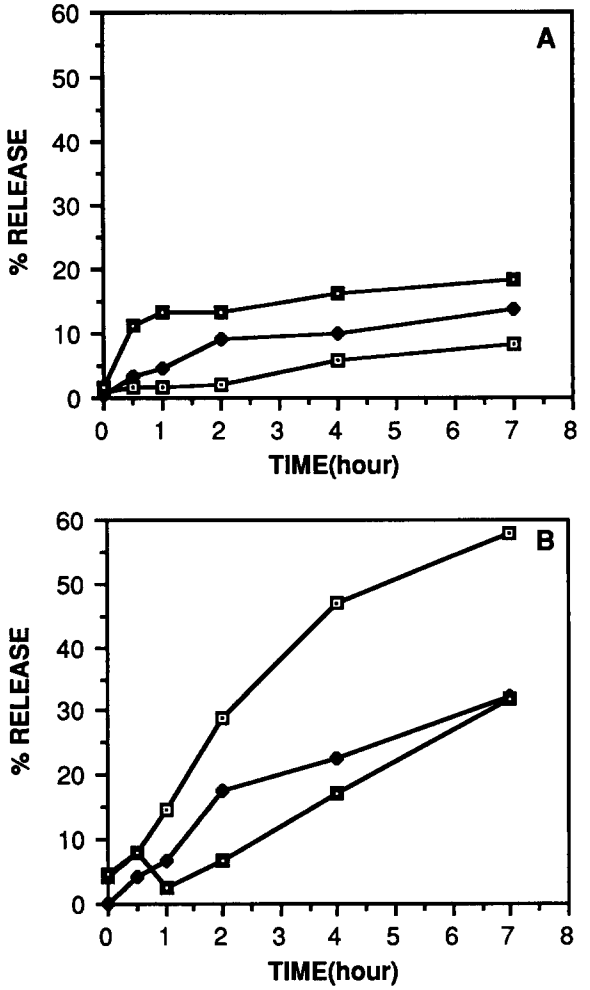

Fig. 3. Effect of GM_1 on stability of DPSG containing liposomes. (A) DOPE/DPSG based liposomes. (B) DOPC/DPSG based liposomes. □, without GM_1; ◆, with 2.5% GM_1; ■, with 5% GM_1.

(Fig. 3B), confirming the observations of Allen et al. [20]. However, the effect of GM_1 on the liposomes composed of DOPE/DPSG (4 : 1) was a slight increase of calcein release during the incubation with plasma (Fig. 3A). The reduction of plasma-stability of the PE-containing liposomes by GM_1 was quite small. Liposomes composed of DOPE/DPSG/GM_1 (76 : 19 : 5) only released about 15% entrapped calcein after 4 h incubation in plasma at 37°C. Although this level of content release was reproducibly higher than that of the same liposomes containing no GM_1 ($\approx$ 5% release at 4 h), both liposome types were essentially plasma stable.

Effect of GM_1 on the biodistribution of liposomes in mouse

The biodistribution of GM_1-containing liposomes in the Balb/c mice was measured at different time periods after i.v. injection. ^{125}I-tyraminylinulin was used as a nonmetabolizable aqueous marker for liposomes. Any free marker leaked from the liposomes is rapidly excreted from the animal leaked from the liposomes is rapidly excreted from the animal with a half-time of a

few minutes (data not shown). Thus, the marker retained in the blood represents intact liposome structures. Inclusion of GM_1 in the liposomes composed of DOPE/DPSG (4:1) had significantly enhanced the amounts of liposomes in the circulation up to 2 hours after injection (Fig. 4). For example, there was $37.9 \pm 3.4\%$ of injected dose in the blood 30 min after injection for liposomes containing 5% GM_1. This is to be compared with $17.1 \pm 0.1\%$ for liposomes containing no GM_1 under the same condition. The difference in blood level among the liposomes containing different amounts of GM_1 diminishes at longer time periods (5 and 24 h).

The uptake of liposomes by liver and spleen of the mice was also measured at different time points (Fig. 5). Statistically significant decreases in the RES (liver plus spleen) uptake of liposomes were found in the early time points for liposomes containing GM_1. There was $33.1 \pm 2.14\%$ of injected dose found in the combined liver and spleen uptake (mainly liver) at 1 h after injection for liposomes containing 5% GM_1, as compared to $47.6 \pm 0.6\%$ for liposomes containing no GM_1 at the same period of time. This decrease in the RES uptake of liposomes was GM_1 dose-dependent, as the values for liposomes containing 2.5% GM_1 generally fell in between the values for liposomes containing 0 and 5% GM_1. At longer time periods (5 and 24 h), the differences in the RES level of liposome uptake diminished.

The data indicate that ganglioside GM_1 can significantly reduce the affinity of PE-containing liposomes to the RES cells and concomitantly increase the level in the blood. However, these effects appeared to be transient as the effect of GM_1 diminished at the longer periods of time.

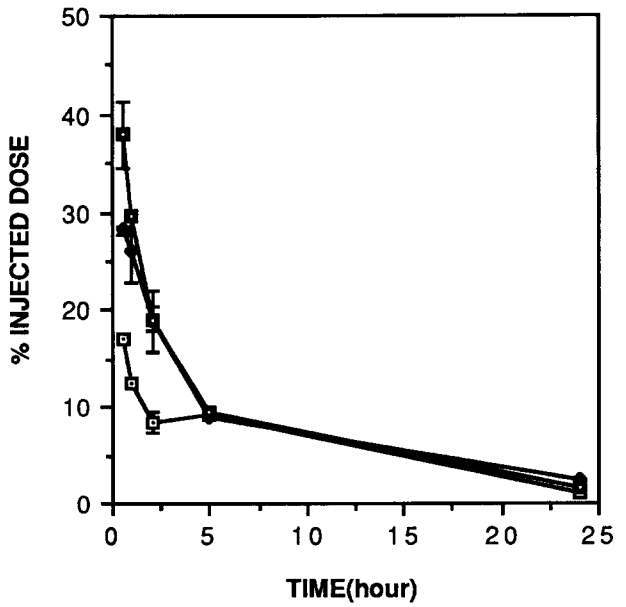

Fig. 4. Retention in blood of liposomes composed of DOPE/DPSG. ⊡, without GM_1; ◆, with 2.5% GM_1; ■, with 5% GM_1.

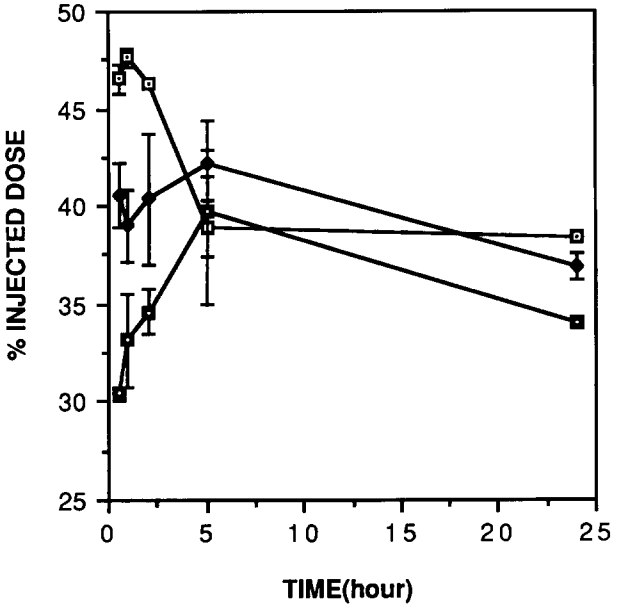

Fig. 5. Time-dependent accumulation in RES (liver and spleen) of liposomes composed of DOPE/DPSG. ⊡, without GM_1; ◆, with 2.5% GM_1; ■, with 5% GM_1.

Discussion

The equilibrium phase of unsaturated PE at physiological conditions is the inverted hexagonal (H_{II}) phase [21]. However, the bilayer (L_α) phase of unsaturated PE can be stabilized by the presence of a weakly acidic amphiphile [2,3,11,22,23]. Protonation of the amphiphile brings about a rapid $L_\alpha \rightarrow H_{II}$ phase transition which destabilizes the liposomes [2,11,22–24]. One of the intermediate states of the phase transition involves membrane contact and the formation of 'inverted micellar intermediate' (IMI) structures at the contact area [25–27]. These intermediate structures give rise to a high fusion activity of the lipid membrane, which is probably the basis of high cytoplasmic delivery activity of the pH-sensitive liposomes after being endocytosed by the target cells. Addition of a second amphiphile such as GM_1, which is not protonated at the weakly acidic pH, further stabilizes the liposome against acidification. We have previously shown that GM_1 itself is a potent bilayer stabilizer for unsaturated PE [28,29]. Indeed, data shown in Fig. 1A and 2 clearly indicate that GM_1 diminishes the liposome's sensitivity to acid in a dose-dependent manner. Therefore, the goal of this study is to search for an intermediate level of GM_1 such that the acid sensitivity of the liposome is partially preserved, yet the liposomes are still stable in plasma and, more importantly, show a reduced affinity to the RES cells in vivo.

We [10] and others [11] have shown that pH-sensitive liposomes composed of unsaturated PE and a double-chain amphiphile are relatively stable in serum or plasma. DPSG is a synthetic lipid which falls in the category of a weakly acidic amphiphile with an ability to stabilize DOPE liposomes against the lysis induced by serum or plasma [10,11]. It was previously assumed that DPSG is not extracted from liposomes by serum albumin, and thus stays in the lipid membrane to stabilize the bilayer phase of DOPE. Our data (Table I), on the other hand, show that DPSG is also transferred to plasma components during incubation with plasma. The degree of transfer was, however, lower than that of a single-chain amphiphile such as oleic acid. Transfer of approx. 70–80% of oleic acid has been reported under conditions similar to those used in this study [8]. Thus, although about 40% of DPSG is extracted from liposomes by plasma, the remaining DPSG is still enough to keep the liposome stable. Furthermore, plasma lipids and/or proteins must also be inserted into the liposome bilayer to help stabilize the liposomes. This is because the acid sensitivity of the plasma-treated liposomes was reduced from that of the original liposomes. Addition of proteins and lipids into DOPE : OA liposomes has resulted in a complete loss of acid sensitivity of the liposomes [8], and these liposomes are very stable in the plasma due to the insertion of plasma components into the liposome membrane [8]. Thus, the situation of DOPE/DPSG liposomes is similar to that of DOPE/OA liposomes, except that the degree of modification of the liposomes by plasma components is less severe in the former case.

The partial acid sensitivity displayed by DOPE/DPSG/GM_1 liposomes is probably sufficient for an effective cytoplasmic delivery of their contents to the target cells. It has been shown that pH-sensitive liposomes containing cholesterol also display a partial acid sensitivity [16], yet these liposomes are efficient intracellular delivery vehicles for a fluorescent dye [5] and DNA [6].

Addition of GM_1 to the PE-containing liposomes has slightly, but reproducibly, reduced the stability of liposomes in plasma (Fig. 3A). This could be due to the proposed activity of GM_1 in reducing the interactions of serum proteins with the liposome membrane [12]. Although the serum proteins involved in such interactions are not yet identified, they are likely to be at least a part of the opsonin system which confers the ordinary liposomes with high affinity to the RES cells (for a recent review, see Ref. 30). Reduction of protein interactions with liposome membrane is probably the mechanism of improved plasma or serum stability of PC-containing liposomes, as previously studied by Allen et al. [20], and also shown here in Fig. 3B. Interactions of plasma components with PE liposomes stabilize the liposomes, whereas the same interactions result in a rapid lysis of the PC liposomes [8]. Thus, GM_1 reduces the plasma stability of DOPE : DPSG liposomes, probably by reducing the interactions of the liposomes with plasma components. Nevertheless, liposomes composed of DOPE/DPSG/GM_1 were still reasonably stable in the plasma (Fig. 3A). The proposed reduction of plasma protein interactions with the liposomes by GM_1 could also be the basis of the observation that the acid sensitivity of liposomes containing high amounts of GM_1 ($\geq 5\%$) was determined by GM_1 and not by the plasma treatment (Fig. 2).

Data shown in Figs. 4 and 5 clearly indicate that GM_1 at least transiently reduces the liposome's affinity to the RES cells and enhances the liposome concentration in the blood. Thus, the 'stealth' activity of GM_1 also works for PE-containing liposomes, although the activity is restricted to the initial few hours after injection. Other reported 'stealth' liposome compositions show longer periods of blood residence [12,14]. It is not clear at the present time why the compositions described here only transiently retard the liposome's uptake by RES. Nevertheless, it is likely that even a small delay in the clearance of liposomes from circulation is sufficient to enhance the level of uptake by the target cells, because liposome binding to the target cells is a relatively rapid process. Immunoliposomes specific for the lung endothelium accumulate in the lung in approximately one minute after i.v. injection (Maruyama et al., unpublished data). Clearance of circulating mouse red blood cells in rats by injecting liposomes covalently coated with anti-mouse red blood cell antibody takes place as rapidly as 10 min after injection [31]. Binding of injected immunoliposomes containing anti-erythrocyte antibody with erythrocytes in the circulation also occurs rapidly (< 15 min after injection) [32]. Thus, a delay of about 2 h in liposome clearance in GM_1-containing liposomes should enhance the chance of liposome binding to the target cells, provided that the target cells are readily accessible by liposomes. Since the DOPE/DPSG/GM_1 liposomes are stable in plasma and are still partially acid-sensitive, they may be an efficient carrier for drugs which need to be delivered to the cytoplasm of the target cells. These hypotheses will be evaluated in suitable animal models.

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