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EFFECT OF GLYCOLIPIDS ON PHOSPHOLIPID INTERACTION AND LIPOSOME CIRCULATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Yong Serk Park

May, 1992

ACKNOWLEDGEMENTS

I would like to express my gratitude to a number of people who have contributed toward the completion of my doctoral research. First of all, I am very grateful to my major professor, Dr. Leaf Huang who has given tremendous support and encouragement for my present and future. His frank advise has nurtured me as a scientist. I am also thankful to my wonderful dissertation committee, Drs. Jorge E. Churchich, Engin Serpersu, and Jeffrey Becker for their advice and encouragement. I am very grateul to a number of my aquaintance: all the folks in Dr. Huang's lab, all the wonderful people in the Department of Biochemistry, and especially all my Korean friends around me.

I wish to express my gratitude to my great family, my parents, brother (Eun Serk) and sisters (Sungmee, Jungmee, and Yumee) for their sacrificing support and sincere understanding. Finally, I am most indebted to my lovely wife (Younghee) who has been always supportive and patient. Her letters over the sea for the last four years always made me feel special and secured. I would like to give my best wishes to my baby who will be born in a few days.

ABSTRACT

A series of glycophospholipids has been synthesized by coupling mono-, di-, or trisaccharides to dioleoylphos-phatidylethanolamine (DOPE) via reductive amination to study effect of glycosyl headgroup on lipid polymorphism. The ability of glycophospholipid to stabilize the DOPE bilayer phase was directly related to the hydration intensity. Glycophospholipids generally reduced the chain-melting temperature (T_m) of dipalmitoylphosphatidylcholine (DPPC). Low level of N-(N-acetylneuraminlactosyl-DOPE (NANL-DOPE) reduced the membrane fluidity of DPPC as well as increased the hexagonal phase transition temperature (T_H) of dielaidoylphosphatidylethanolamine (DEPE); whereas the other glycophospholipids did not. Trisaccharide-DOPE, i.e. N-isomaltosyl DOPE and NANL-DOPE had similar activity in cryoprotection, whereas mono- and disaccharide-DOPE, i.e. N-galactosy-DOPE and N-lactosyl-DOPE, did not show any activity, and even reduced the cryoprotective effect of trehalose, a well-known cryoprotectant.

To investigate the effect of glycosyl headgroup on liposome biodistribution, several derivatives of phospholipid or glycolipid were synthesized: (1) a series of negatively charged phospholipid derivatives, (2) ganglioside G_{M1} derivatives in which the sugar group had been modified by periodate oxidation, reduction, or reductive amination, (3) G_{M1} derivatives in which the negative charge of sialic acid has been removed by methylation or reductive hydrolysis, (4) neoglycolipid analogs of G_{M1} synthesized by coupling the G_{M1} oligosaccharide to DOPE via different spacer arms. The derivatives were incorporated into egg PC/ cholesterol liposomes to test their effects on prolonging the liposome circulation. Only N-glutaryl DOPE

and N-adipyl DOPE were effective in prolonging the liposome circulation. The oxidized G_{M1} was ineffective in prolonging the liposome circulation and however, the lost activity was completely recovered by subsequent reduction or some reductive amination. Removal of the negative charge of G_{M1} did not deprive of its activity. Among the neoglycolipid analogs of G_{M1}, only G_{M1} oligosaccharide directly conjugated to DOPE was effective in prolonging the circulation. Lipid A was included in the liposomes containing G_{M1} or poly(ethyleneglycol)-PE (PEG-PE) and its effect on the liposome biodistribution was also investigated. Lipid A, being an opsonizing lipid, more effectively antagonized G_{M1}'s activity than PEG-PE's in prolonging the liposome circulation. This may be related to the different mechanisms exerted by G_{M1} and PEG-PE. These results indicate that the molecular structures of the negatively charged DOPE derivative and G_{M1} oligosaccharide are very important for their functional activities in prolonging the liposome circulation,which may be regulated by putative "dysopsonin(s) ".

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LIST OF ABBREVIATIONS

DCC	dicyclohexylcarbodiimide
DEPE	dielaidoylphosphatidylethanolamine
DOPE	dioleoylphosphatidylethanolamine
DPH	1,6-diphenylhexatriene
DPPC	dipalmitoylphosphatidylcholine
DSC	differential scanning calorimetry
DSS	disuccinimidyl suberate
DTPA-SA	diethylenetriamine pentaacetic acid distearylamine complex
FITR	Fourier transofrm infrared spectroscopy
Gal-DOPE	N-galactosyl-DOPE
G_{M1}	monosialoganglioside, II^3Neu5Ac-GgOse$_4$Cer
G_{M1}OS	oligosaccharide portion of G_{M1}, II^3Neu5Ac-GgOse$_4$
G_{M1}OS-NH_2	1-amino-1-deoxyglucitol derivative of G_{M1}OS
G_{M1}OSNH-X-DOPE	suberyl DOPE conjugated to $G_{M1}OSNH_2$
G_{M1}OSNHC8-NH-X-DOPE	suberyl DOPE conjugated to G_{M1}OS via diaminooctane
H_{II} phase	reverse hexagonal phase
HPTLC	high performance thin layer chromatography
IMT-DOPE	N-isomaltotriosyl-DOPE
i.v.	intravenous
L_α phase	liquid-crystalline phase
L_β phase	gel phase
Lac-DOPE	N-lactosyl-DOPE
LPS	lipopolysaccharide

NANL-DOPE	N-(N-acetylneuraminlactosyl)-DOPE
NAPE	N-adipyl DOPE
NGPE	N-glutaryl DOPE
NMPE	N-malonyl DOPE
NPPE	N-pimelyl DOPE
NSPE	N-succinyl DOPE
NSBPE	N-suberyl DOPE
oxidized$C7G_{M1}$	a G_{M1} derivative containing a C7 analog of sialic acid (5-acetamido-3,5-dideoxy-6-aldehyde-hexulosonic acid)
PC	phosphatidylcholine
PEG-PE	poly(ethyleneglycol) dioleoylphosphatidylethanolamine
PI	phosphatidylinositol
reduced $C7G_{M1}$	a G_{M1} derivative containing a C7 analog of sialic acid (5-acetamido-3,5-dideoxy-heptulosonic acid)
reduced $C9G_{M1}$	a G_{M1} analog having a hydoxymethyl group instead of the carboxyl group of sialic acid
RES	reticuloendothelial system
T_H	transition temperature from L_α to H_{II} phase
T_m	transition temperature from L_β to L_α phase

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PART I

INTRODUCTION

Ever since the initial reports describing liposomes as a model for biological membranes and the ability of liposomes to entrap hydrophilic small molecules (1,2), liposomes have been utilized and developed for many applications in biology and medicine. Liposomal vesicles are excellent model system for biophysical studies of biological membranes. They are also used to deliver a wide range of therapeutic or diagnostic agents in experimental animals and humans. The physical properties of liposomes (3) and the pharmaceutical applications (4) have been described in a number of excellent reviews.

1. Liposomes

Liposomes are closed vesicular structures composed of lipid bilayers which are spontaneously assembled from suspending phospholipid components in an aqueous environment. Liposomes have been classified into three types on the basis of size and lamellarity: multilamellar vesicles (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV). Lamellarity, the number of internal lamellar sequestered within vesicles, is usually determined by freeze-fracture microscopy (3). Light scattering techniques and electron microscopy are used to measure the vesicle diameter.

MLVs are vesicles having a morphology consisting of concentric and alternating phospholipid bilayers and aqueous compartments. Mechanical dispersion of a lipid film results in the spontaneous formation of MLV in an aqueous solution. MLV are heterogeneous in size (0.2-10 μm) and lamellarity. While preparation of MLV is relatively easy, the heterogeneity in size and lamellarity limits their general use as a pharmaceutical

formulation. LUV have a well-defined large internal aqueous compartment surrounded by a single lipid bilayer and are relatively homogeneous in size (50-400 nm). Several procedures have been developed to prepare LUV, i.e. reverse phase evaporation (5), detergent dialysis (6), and medium pressure extrusion (7). Using the extrusion method, homogeneous LUV composed of a variety of lipid species can be produced in large quantity. SUV have a small internal compartment (20-50 nm in diameter) surrounded by a single bilayer. SUV are prepared by sonicating MLVs (8) or by French press technique (9). SUV have a highly strained membrane curvature which produces a tendency for the vesicles to fuse (10,11).

2. Lipid polymorphism

A great deal of effort has been spent to elucidate polymorphism of lipid bilayer (for review, see refs 12,13). Phospholipid aggregates dispersed in an aqueous system can be classified into the bilayer phase and nonbilayer phase. The bilayer phase can be also divided into gel phase ($L\beta$) and liquid crystalline phase ($L\alpha$) according to the acyl chain ordering. The nonbilayer phase includes inverted hexagonal phase (H_{II}), micelle phase (H_I), cubic phase, and others. The sequence of thermotropic transition for phospholipids that exhibit limiting hydration is as follows:

$$\underset{L_\beta}{\overset{T_p}{\dashrightarrow}}\ \underset{L_\beta'}{\overset{T_m}{\dashrightarrow}}\ \underset{L_\alpha}{\overset{T_H}{\dashrightarrow}}\ H_{II}$$

Where T_p, T_m, and T_H represent pretransition, chain melting, and hexagonal phase transition temperatures, respectively. A change in specific factors such as hydrocarbon unsaturation, temperature,

headgroup size, headgroup ionization, water content, etc., affect the preference of phospholipids for a bilayer or a H_{II} configuration.

Phospholipids are classified into 3 categories depending on their equilibrium structure in an aqueous environment (left column in Figure 1-1). Most of the glyceryl phospholipids including PE, PC, PS, PG, PI, and sphingomyelin readily form stable bilayers in an aqueous medium at the physiological pH, ionic strength, and temperature. However, single chain lysophospholipids, some polar lipids such as glycolipids, and fatty acids readily aggregate to form micelle (H_I) phase under physiological conditions. The last class of lipids is the unsaturated PE which readily aggregates to form nonbilayer structures such as the inverted hexagonal (H_{II}) phase and inverted cubic phase. The classification of phospholipids has been well reviewed with the introduction of polymorphic phases and the corresponding dynamic molecular shapes of the component lipids (13).

Individual phase of phospholipid has been studied using several different techniques. X-ray diffraction is fundamentally important in elucidating the structure of lipid phases (14). Different phases show characteristic pattern of symmetry with the small angle X-ray diffraction, which arises from the periodic placement of the lipid molecules on a lattice, one-dimensionally (lamellar phase), two-dimensionally (H_I and H_{II} phase), and three-dimensionally periodic phase (cubic phase). The characteristic X-ray diffraction pattern, i.e. ratio of the radii of a series of diffraction lines, gives useful information on the lipid structure. Secondly, the nonbilayer structure has been identified by the use of ^{31}P-NMR (12,15). The differences in the shape of the spectra result from the degree of diffusional freedom of the phospholipid molecule in the lipid aggregate (middle column on Figure 1-1). The rapid axial rotation in the lamellar

structure produces motional averaging, which results in the projection of the anisotropic shielding along the axis of rotation. This chemical shift anisotropy results in the asymmetric ^{31}P-NMR lineshape spectra. In H_{II} system, additional motional averaging, resulting from lateral diffusion of the lipid around the aqueous cylinders, produces the narrower lineshape with reversed asymmetry. The isotropic motional averaging of the cubic or micelle phase leads to a narrow, symmetric ^{31}P-NMR lineshape. Finally, freeze-fracture electron microscopy has been especially important in elucidating lipid polymorphism (16). Lamellar structures yield smooth fracture faces, whereas the fracture face of H_{II} phase appears as a series of parallel, concentric ridges arranged in distinct layers (right column in Figure 1-1).

The head group of PE is poorly hydrated compared to that of other bilayer-forming phospholipids. The poor hydration of PE contributes to the low intermolecular hydrogen bonding between the head groups of PE. Unsaturated acyl chains containing cis double bonds expand the volume occupied by the hydrocarbon chains. The combined effects of the headgroup and acyl chain provide an unique physical property to the unsaturated PE, i.e. dioleoyl PE (DOPE), the ability to assume the H_{II} phase conformation. While pure DOPE is not able to form stable bilayers under the physiological conditions, DOPE can be stabilized in the bilayer structure in the presence of a stabilizer, e.g. PS (17), PC (18), PG (18), lysoPC (18,20), and glycolipids (19). Protein such as glycophorin (21) and fatty acid derivatized antibodies (22) can also stabilize DOPE in the bilayer form.

3. Glycolipid polymorphism

The glycolipids are a structurally diverse group of membrane components found from bacteria to animal cells. To understand the function of glycolipids, three general approaches have been taken: analysis of glycolipids in different cells, physical behavior of glycolipids in the cell membrane, and biochemical functions of glycolipids. Among these approaches, the physical behavior of glycolipids has been elucidated by using various structural and spectroscopic means, e.g. differential scanning calorimetry, X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, electron spin resonance (ESR) spectroscopy, and electron microscopy.

Glycolipids of bacteria and plants are generally glycosyldiacylglycerols, e.g. monogalactosyldiacylglycerol (MgalDG), monoglucosyldiacylglycerol (MGlcDG), digalactosyldiacylglycerol (DGalDG), and diglucosyldiacylglycerol (DGlcDG). The major glycolipids of animals consist of a mono- or oligosaccharide linked to ceremide, e.g. galactosylceremide, glucosylceremide, and lactosylceremide. Gangliosides are ceremide-based glycolipids which possess N-acetylneuraminic acid (NANA) derivatives. These anionic glycosphingolipids appear to be ubiquitous in mammalian membranes, but are found only in low concentration.

a. Glycosyldiacylglycerols

It has been reported that MGalDG with 5 or more double bonds per molecule forms a H_{II} phase, while MGalDG with 4.5 or fewer double bonds per molecule forms a lamellar phase (23). Freeze-fracture electron

microscopy and X-ray diffraction studies showed that equimolar mixtures of the native and hydrogenated MGalDGs exhibited both lamellar and H_{II} phases at 29 °C. The minimum conformational energy calculation indicated a conical structure for MGalDG, with the glycosyl headgroup at the small end of the cone (24). The computed structure of DGalDG was cylindrical consistent with the appearance of a lamellar phase. Thus, unlike the MGalDG, the DGalDG forms only lamellar phases, regardless of the extent of acyl chain unsaturation (25). MGalDG showed a higher T_m than PC of similar acyl chain composition and a metastable gel state below the T_m, which implies the existence of interlipid hydrogen bonding via the hydroxyls of glycosyl residue (26). The T_m of Di-18:0-DGalDG was approximately 55 °C, a temperature similar to the T_m of di-18:0-PC (25).

Electron microscopy of the mixtures of MGalDG and DGalDG reveals the presence of a lamellar phase containing 'lipidic particles' which are believed to consist of inverted micelles within the lipid bilayers (27). The addition of ethylene glycol induced the formation of the lipidic particles, which implied that lipid-lipid or lipid-water hydrogen bonding is an important determinant of the phase behavior of these glycolipid mixtures (28). ^{2}H-NMR and ^{19}F-NMR spectroscopy have been used to study the orientational order of the acyl chains in *A. laidlawii* membranes which are enriched with glycosyldiglycerol. Specifically, deuterated dipalmitoyl PC bilayers exhibited a similar acyl chain order profile to glycosyldiacylglycerol (29), which implies that acyl chain ordering in glycosyldiacylglycerol is probably similar to that in phospholipids.

b. Neutral glycosylceremides

The crystal structure of a synthetic Gal-Cer showed that the Gal-Cer molecules were packed in a bilayer configuration, with the plane of the sugar ring almost parallel to the bilayer plane (30). The most significant feature of the crystal structure appeared to be the presence of extensive hydrogen bonding among the polar galactosyl headgroups, the N-H and hydroxyls of sphingosine backbone, and the hydroxy fatty acid (30). ^{2}H-NMR studies of 10:0-nonhydroxy fatty acid (NFA)-Gal-Cer indicated restricted mobility in the gel and liquid-crystalline structure (31). The X-ray studies on 16:0-NFA-Gal-Cer have suggested that two types of gel state, i.e. crystal form A and E exist and conversion of the metastable form A to the stable form E involves the hydration of its sugar headgroup (32). The hydroxylation on the acyl chain appears to provide a kinetic barrier between state A and E, which suggested that a function of hydroxy fatty acid is to prevent metastable dehydration (33). The extensive interlipid hydrogen bondings seem to mediate the metastable behavior of sphingolipids as well as the exceptionally high T_m.

Very little is known about the detailed phase behavior of neutral glycosphingolipids with two or more sugars. As the number of sugar groups in the glycosyl headgroup increases, the temperature and enthalpy of the order-disorder transition decrease. The effect of acyl chain length is small compared to the effect of polar headgroup complexity (34). While Gal-Cer forms a lamellar phase in water, tetraglycosylceremide forms cylindrical micelles (35).

Glycosphingolipids appear to form small clusters in a liquid-crystalline membrane due to their extensive hydrogen-bonding capability.

The lectin labeling/freeze etch approach provided useful information on glycolipid distribution in membranes. Using ferritin-conjugated Ricinus communis agglutinin to label the asialo-G_{M1} in the PC liposomes made it possible to localize the troughs of the rippled PC surface below Tm and the clusters in the membrane plane above Tm (36-38). Clustering of asialo-G_{M1} in liquid-crystalline bilayers was supported by the studies of biphasic transfer of asialo-G_{M1} from donor SUV to acceptor LUV of PC in the liquid-crystalline state (39).

Some spectroscopic studies have been carried out to characterize the order and dynamics of neutral glycosphingolipids. Electron spin resornance (ESR) studies on the PC bilayers containing a small amount of spin-labeled globoside indicated that the motion of the terminus of the globoside glycosyl headgroup is affected by the acyl chain behavior of the PC matrix (40). ^{2}H-NMR spectra of the labeled 16:0-NFA-Gal-Cer indicated that the glycosyl group is quite rigid in the gel phase, and undergoes only very slow motions in the liquid-crystalline phase (41). These observations are consistent with the presence of interlipid hydrogen bondings involving the glycosyl headgroups. ^{13}C-NMR spectra of labeled 18:1NFA-Gal-Cer/egg PC (42) and ^{2}H-NMR spectra of labeled di-16:0 PC/Gal-Cer (43) showed that the glycolipids induced the acyl chain order in the liquid-crystalline phase.

c. Gangliosides

Mixed brain gangliosides form a H_I mesophase at low water contents (18-50 wt% H_2O) and micelles at high water contents (over 60 wt% H_2O) (44). The incorporation of G_{M1} into PC below 30 % forms bilayers, a mixed micelle phase above 75 %, and an intermediate phase at intermediate G_{M1}

content at room temperature (45). PC can be solubilized by anionic gangliosides and the more highly charged gangliosides are more efficient solubilizers of the PC bilayers (46). Freeze-etch electron-microscopy has been used to locate the ganglioside clusters in PC/G_{M1} mixture. Peters et al. (38) have shown that G_{M1} partitions in both gel and liquid-crystalline phase of the PC bilayers and self-associates in clusters. However, Thompson et al. (36) have reported that G_{M1} is evenly dispersed in liquid-crystalline of PC bilayers over the range of 0-5 mol% G_{M1} and does not aggregate in clusters. They have also claimed that G_{M1} clusters may be formed during slow freezing process used in the freeze-fracture EM. ^{13}C-NMR and ^{1}H-NMR studies have showed that the mobility of the acyl chain of gangliosides in micelles is slower than that of PC bilayers (47). ESR studies of spin labeled gangliosides in egg PC bilayers showed a cooperative glycosyl headgroup interaction resulting in decreased mobility (48). With increasing ganglioside content, the fluidity of the apolar bilayer interior decreased (46). The effect of G_{M1} on the membrane fluidity was slightly greater than those of G_{D1a} and G_{T1b}.

4. Cryopreservation of liposomes

The integrity of biological membranes and phospholipid bilayers can be maintained only in the aqueous medium (49). When water is removed, the lipid molecules rearrange their aggregation pattern and irreversible changes occur in the membrane structure and function (50). The stability of the biological membrane against dehydration condition is very important for the survival of the organism in extreme dehydrated environments. However, in recent years evidence has been accumulating that certain

sugars may replace water around the polar residues in membrane phospholipids, thereby maintaining their integrity in the absence of water. Many anhydrobiotic organisms, i.e. fungal spores and yeast cells, contain large quantities of trehalose (for review, see ref. 51). In pollen and seeds of higher plants, sucrose, which shares many of the properties of trehalose, appears to function as a cryoprotectant.

Cryopreservation of liposomes is important for the design of a liposomal formulation to maximize stability under appropriate storage conditions. Aqueous suspensions of liposome are expected to exhibit drug leakage and possibly lipid degradation during storage. For this reason, liposomal systems which can be lyophilized for prolonged storage and readily reconstituted by rehydration are highly desirable.

a. Preservation of dry phospholipid bilayers

Trehalose, sucrose, and some sugar alcohols can prevent massive fusion of PC bilayers and release of entrapped material under a dehydration condition (52-55). When unilamellar vesicles are freeze-dried and rehydrated, they leaked all their contents to the medium. When similar vesicles are freeze-dried in the presence of trehalose, however, they can be sufficiently protected that over 90 % of the trapped solute is retained upon rehydration. Disaccharides are in general superior to other monosaccharides or oligosachharides, but there is also considerable variability among the disaccharides; trehalose is the best and sucrose is the next.

A great number of studies have been designed to elucidate the mechanism of stabilization of liposomes by sugars (for review, see ref. 56).

Effect of sugar on inhibition of fusion during dehydration have been studied, using freeze-fracture and resonance energy transfer. About 70 % lipid intermixing occurred following freeze-drying and rehydration, whereas lipid intermixing was stopped in the presence of trehalose and other disaccharides (52). Effect of sugar on the thermotropic phase transition of dry unilamellar vesicles have been investigated using differential scanning calorimetry (DSC) method. Cryoprotectant sugars have profound effect to depress the main transition temperature (T_m) of PC vesicles (Lα to Lβ phase) (57,58). It is known that lipid vesicles are most leaky when their membranes undergo a phase transition. Based on these observations, it has been suggested that the ability of a sugar to depress T_m is critical for its activity to stabilize membranes and phospholipid vesicles such that the membranes are maintained in the liquid crystalline state even they are dehydrated. When the membranes are rehydrated, they do not undergo a transition from gel to liquid crystalline phase, thus spared from leakage.

b. Mechanism of interaction between sugar and dry phospholipid

A number of studies support that the stabilization of phospholipid vesicles by sugar requires direct interaction between the sugar and the phospholipid head group (55,58,59). Addition of europium ions, which form a strong ionic linkage with the phosphate groups of membrane phospholipids, to unilamellar phospholipid vesicles made in the presence of sucrose abolishes the ability of sucrose to inhibit membrane fusion during freezing (58). Additional supporting evidence has been obtained by infrared spectroscopy. In dry phospholipids, trehalose strongly affects the bands

assigned to the phosphate group; the P=O asymmetric stretch is displaced to a lower frequency (51). Addition of water to the dry lipid has much the same effect. Furthermore, bands assigned to -OH vibrations in the trehalose molecule are also affected by the presence of the lipid (51). From these studies, it has been suggested that the phosphate group of the phospholipid interacts with -OH groups of the trehalose, probably by hydrogen bonding.

c. Effects of sugars on hydrated phosphatidylethanolamine

It is well established that sugar can stabilize PC vesicles during freezing and direct interaction between sugar and the PC head group is responsible for the stabilization (55,58,59). However, there has been controversy in effect of sugar on the T_m and T_H of phospholipid vesicles composed primarily of PE. Strauss et al. have reported that sugars are capable of elevating the T_m of hydrated vesicles (55). Rudolph et al. have reported that trehalose elevated the T_m of MLV but decreases the T_m of LUV (57). Wistrom et al. have claimed that trehalose is capable of maintaining DOPE in lamellar phase at temperatures well above T_H (60). However, Bryszewska et al. have shown that a number of sugars including trehalose lowered the T_H of DEPE (61). Further investigation is needed to resolve the controversy.

d. Glycolipid stabilizing phospholipid vesicles

Goodrich et al. have found that sugar residues covalently bonded to cholesterol via oxyethylene are capable of stabilizing DPPC vesicles as well as depressing the T_m of DPPC (62,63). They hypothesized that the effect of

the glycolipid could be due to the coordinate hydrogen bonding of the sugar residue with the neighboring phospholipid head groups.

5. Fate and behavior of liposomes in vivo

Liposomes are rapidly cleared from the circulation in a biphasic manner, with an initial rapid phase followed by a slow rate of elimination (64). A great number of studies have established that liposome clearance from circulation results from a combination of the destabilization of liposomes by plasma components and the uptake of liposomes by the resident macrophages of the liver and spleen (for review, see refs 65,66). These two subjects will be separately reviewed.

a. Destabilization of liposomes

It has been suggested that leakage of the liposomes entrapped solute in the circulation is plasma-dependent (67). Liposomes become leaky upon incubation at 37 °C in the presence of plasma. Most investigators employ reporter molecules such as a fluorescence marker or a radioactive marker entrapped inside the liposomes. Net transfer of liposomal lipids to the lipoproteins has been shown to destabilize liposomal membranes resulting in the leakage of the entrapped solutes (67). Liposomes interact primarily with the high density lipoproteins (HDL) (68). Using ^{3}H- or ^{14}C-labeled egg PC liposomes containing [^{125}I]albumin, it has been shown that rat, monkey, and human HDL take up the liposomal phospholipids resulting in a massive release of [^{125}I]albumin. Other lipoproteins have also been related to the destabilization of liposome membranes. Net transfer of [^{14}C]PC from

egg PC SUV to low density lipoproteins (LDL) has also been studied. Some anionic liposomes, i.e. PG/chol liposomes, are more unstable in the presence of LDL than in the presence of HDL. However, incorporation of cholesterol to PC liposomes inhibits the transfer process and stabilizes the liposomes in the presence of plasma components. ApoA1 of HDL is believed to be responsible for the recognition of liposomal phospholipids and ApoA1 binding to the liposomes may be dependent on the phospholipid composition of the liposomes (69).

Activation of the complement system is another possible mechanism for the liposome destabilization in circulation. Recently, a number of studies have focused on the liposome lysis by the complement components (70-72). Activation of the complement system results in the formation of a membrane attack complex which induces a local phospholipid disorder and leads to cell lysis (73). Liposome lysis induced by the complement activation is dependent on the liposomal composition and the surface charge content of the liposomes.

b. Interaction of liposomes with cells

Although there is wealth of data about the clearance and deposition of liposomes in vivo, the detailed mechanism of liposome interaction with tissues and cells remains unclear (for review, see refs. 74,75). Most liposomes administered i.v. are rapidly cleared from circulation by the free and fixed macrophages of the reticuloendothelial system (RES) and concomitantly localized mainly in the liver and spleen (for review, see refs. 65,67). Different routes of administration other than the i.v. route reduce the rate of uptake of liposomes by the liver and spleen, but eventually they

still end up in the RES cells in these organs. The behavior of liposomes in circulation can be affected to a considerable extent by manipulating certain structural and compositional parameters of the liposomes such as lipid composition, vesicle size, injection dose, and surface characteristics including charge, hydrophobicity, and the presence of homing devices such as antibodies. It has been recognized that size and surface charge are critical factors in the clearance of liposomes (76). Small liposomes are cleared more slowly than larger ones. Positively charged and neutral liposomes circulate longer than negatively charged ones of similar size. Giant liposomes (over 12 μm in diameter) are trapped in the capillary bed of the lungs first encountered by liposomes upon injection into the blood stream (77). Small liposomes (less than 0.1 μm) are able to pass through the fenestra of the liver sinusoids and interact with liver parenchymal cells (hepatocytes) and avoid the phagocytic cells, i.e. Kupffer cells (78,79). This seems to be the main reason why large liposomes which are taken up by the Kupffer cells are degraded in the liver about three times faster than SUV (80). The total amount of liposomal lipid administered is also an important factor for the clearance rate and tissue distribution. Injection of larger doses slows down the clearance rate due to saturation of the RES (81). In addition to size and dose, the charge of liposomal surface and the liposomal composition affects the liposome circulation and their biodistribution. PS, PG, and other negatively charged phospholipids enhance liposome uptake by the RES cells (82,83). Incorporation of cholesterol or sphingomyelin into liposomes prolongs the circulation time (67). Recently, it has been reported that ganglioside G_{M1} (84) and polyethyleneglycol (85) containing liposomes have a prolonged circulation time.

In addition to the uptake of intact SUV by hepatocytes, there are some

evidence of liposome uptake by cells other than the RES. Increased accumulation of liposomes, especially small ones, has been reported to occur at site of inflammation (86) and in tumor (87,88). From the point of view of drug delivery, the mechanisms by which liposomes deliver their contents to the target cells has been studied (for review, see ref. 89). Several possible mechanisms of liposome-cell interactions are presented in Figure 1-2 (90). Uptake usually takes place by nonspecific endocytosis which can be increased by linking ligands, which are recognized by the receptors on the target cells, to the liposome surface (Figure 1-2a). The fusion mechanism, which is a very attractive route for delivering entrapped contents to cells, has been described for the fusogenic liposomes, i.e. liposomes with viral fusion protein (91), but is a rare event for the nonfusogenic liposomes (Figure 1-2b). In addition to endocytosis, stable surface adsorption is is another major mechanism of liposome-cell interaction (Figure 1-2c) (92). The entrapped contents of the adsorbed liposomes may be released at the cell surface, resulting in a transiently high concentration of the contents at the adsorption site (93). Finally, exchange of lipid molecules may take place between the liposomes and the cell membrane (Figure 1-2d). Hydrophobic drugs incorporated into liposome membranes may also be translocated to the cell membranes. However, there is no substantial evidence to support the lipid exchange mechanism when liposomes encounter cells in vivo.

c. Effect of glycolipids on liposome-cell interactions

Glycolipids are involved in many different biological processes, e.g. cellular recognition, cell surface receptors, and others (for review, see ref. 93). Some glycolipids have been incorporated into liposomal membranes to

modify the biological properties of liposomes. Recently, it has been reported that ganglioside G_{M1} is capable of reducing the rate and extent of liposome uptake by the RES, thereby prolonging the circulation time in vivo (94,95). Longer circulation time of liposomes offers viable therapeutic opportunities. Interaction of cholera toxin with G_{M1} is relatively well understood (96). However, the RES avoidance mechanism of G_{M1} is not clear at all.

Another glycolipid used in the therapeutic applications of liposomes is lipid A which is the lipid portion of lipopolysaccharide (LPS) of Gram-negative bacterium. LPS has numerous biological activities, some toxic and some beneficial, which include the induction of the characteristic endotoxin shock syndrome (97), the nonspecific activation of the immune system (98), and the activation of the complement cascade (99). LPS and lipid A activate macrophages to a tumoricidal state by inducing the production of interleukin-1 (IL-1), tumor necrosis factor alpha (TNF), and other cytokines. They also enhance the humoral immunity to a wide range of haptens and antigens (for review, see refs. 98,100,101). Because of its potent endotoxic activities, lipid A by itself has a limited value as an adjuvant in human vaccines. However, incorporation of lipid A into liposomes greatly reduces many of the toxic effects normally associated with the endotoxin. Therefore, liposomes containing lipid A could be used as a potential carrier for vaccines (for review, see ref. 102). There is a controversy as to whether liposomes enhance or suppress the potency of lipid A to activate macrophages. Some groups have shown that the encapsulation of LPS or lipid A within liposomes enhances its ability to activate murine alveolar and peritoneal macrophages to become tumoricidal (103,104). Other groups have claimed that the incorporation of LPS into liposomes reduces the activation ability of LPS using RAW 264.7

macrophages and murine peritoneal macrophages (86,105). Further investigations are needed to resolve the discrepancy.

6. Organization of The Dissertation Project

The first goal of this project is to deepen our understandings of the biophysical function of the sugar moiety of glycolipid. Artificial glycolipids with defined sugar structures have been synthesized. Their cryoprotectant activities are studied in a liposomal model system. The second goal is to investigate the effect of glycolipid on the fate of liposomes in vivo and the underlying mechanisms of action. Structure of the synthetic and naturally occurring glycolipids studied in this project are shown in Figures 1-3,1-4, and 1-5. Major contributions of this work are: 1) the elucidation of the effect of glycolipids on the lipid polymorphism, 2) the demonstration of cryostability of sugar conjugated to phospholipids, 3) the elucidation of glycolipid effect on the liposome circulation and in vivo deposition, and 4) the suggestion of a hypothetical mechanism involved in the prolongation of the liposome circulation time.

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Appendix

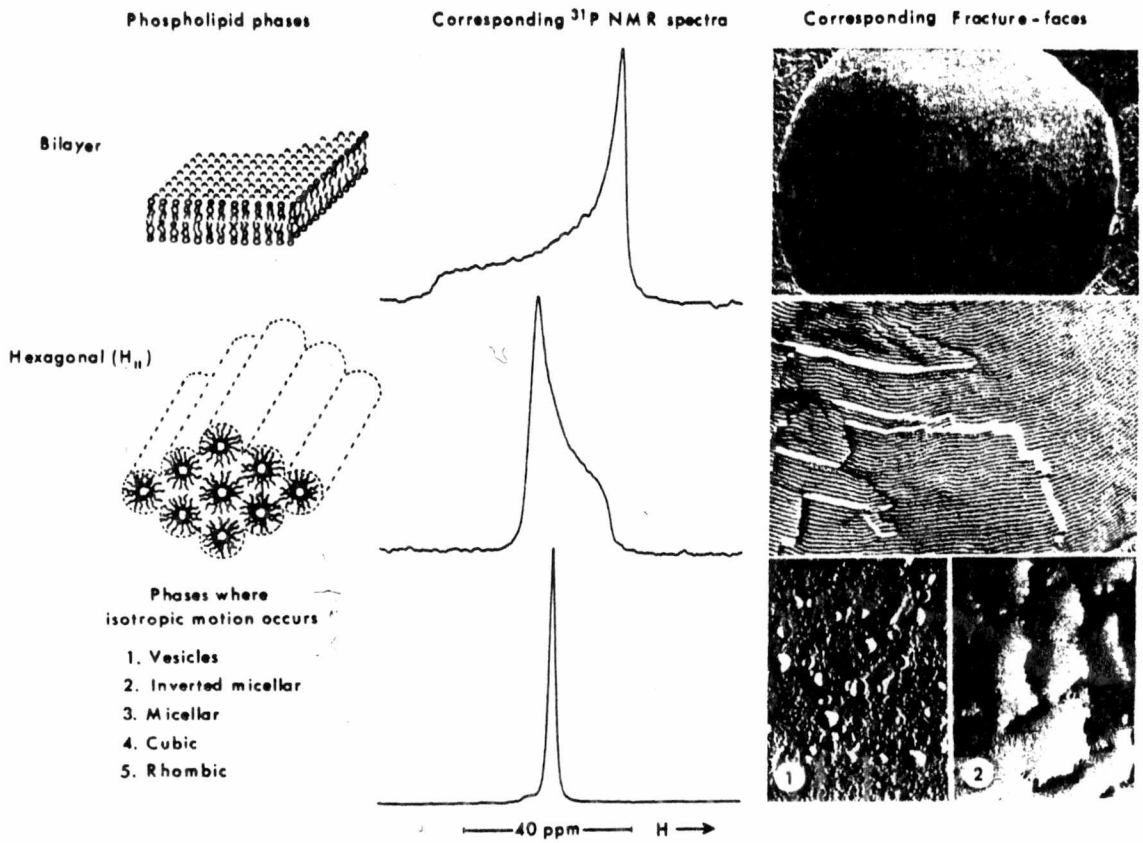

Figure 1-1. Various phospholipid structure with their typical 31P-NMR spectra and freeze-fracture faces are observed for these structure.

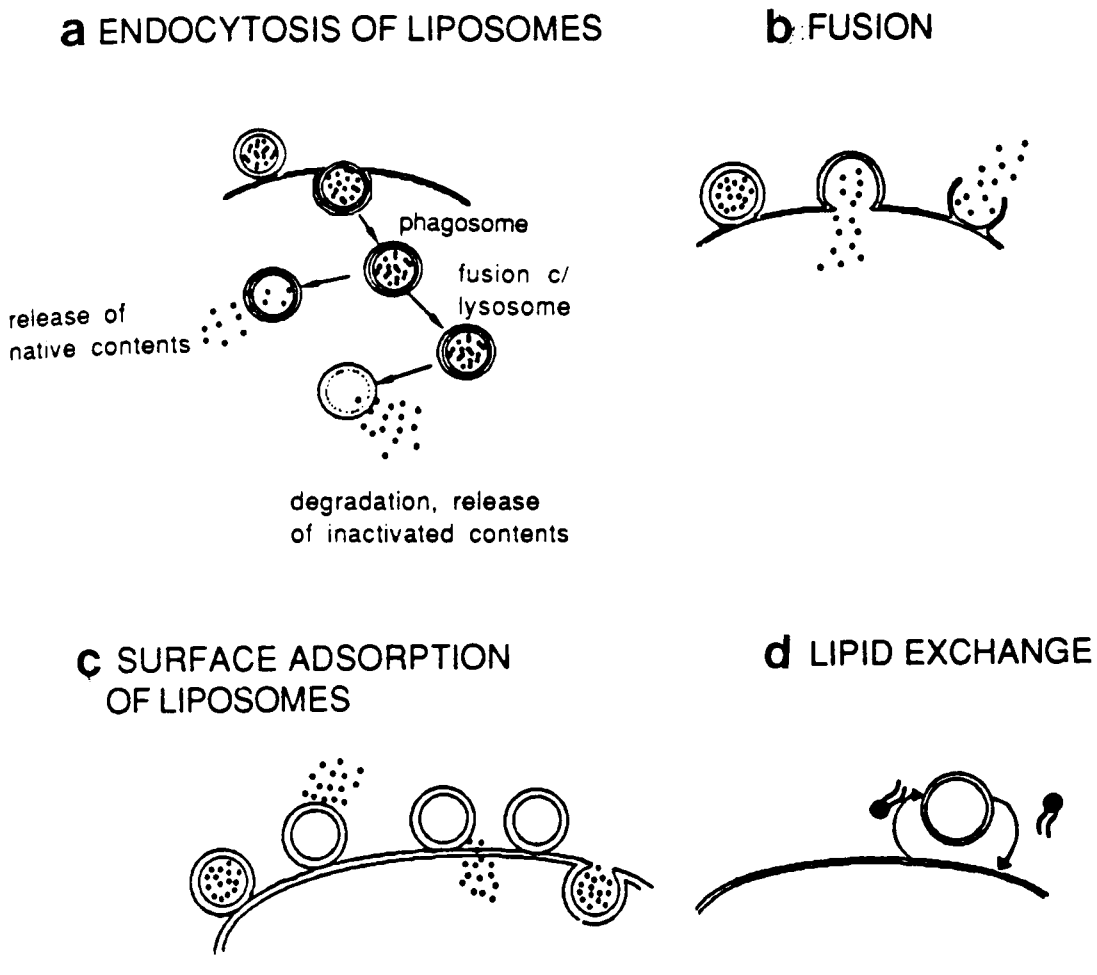

Figure 1-2. Various mechanisms of liposome-cell interaction (modified from Pagano and Weinstein, 1978)

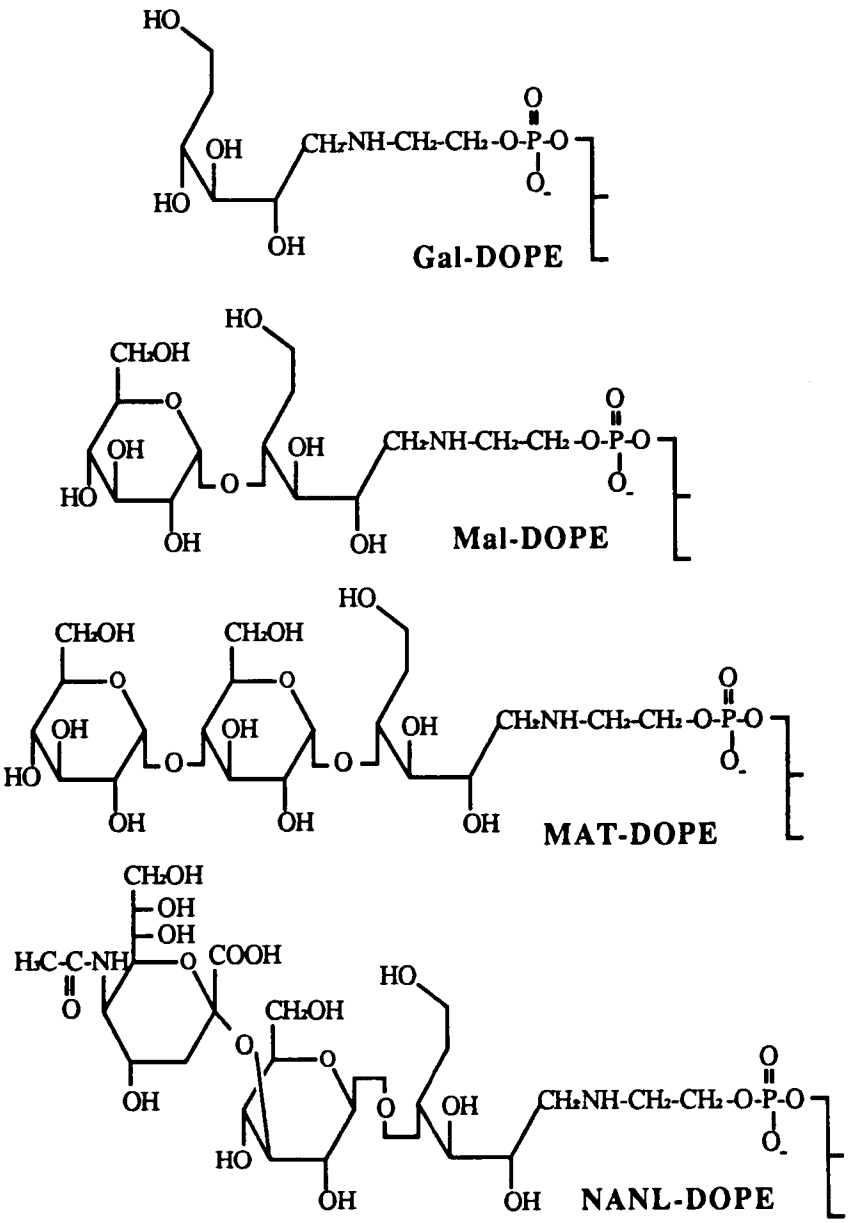

Figure 1-3. Structures of glycophospholipids.

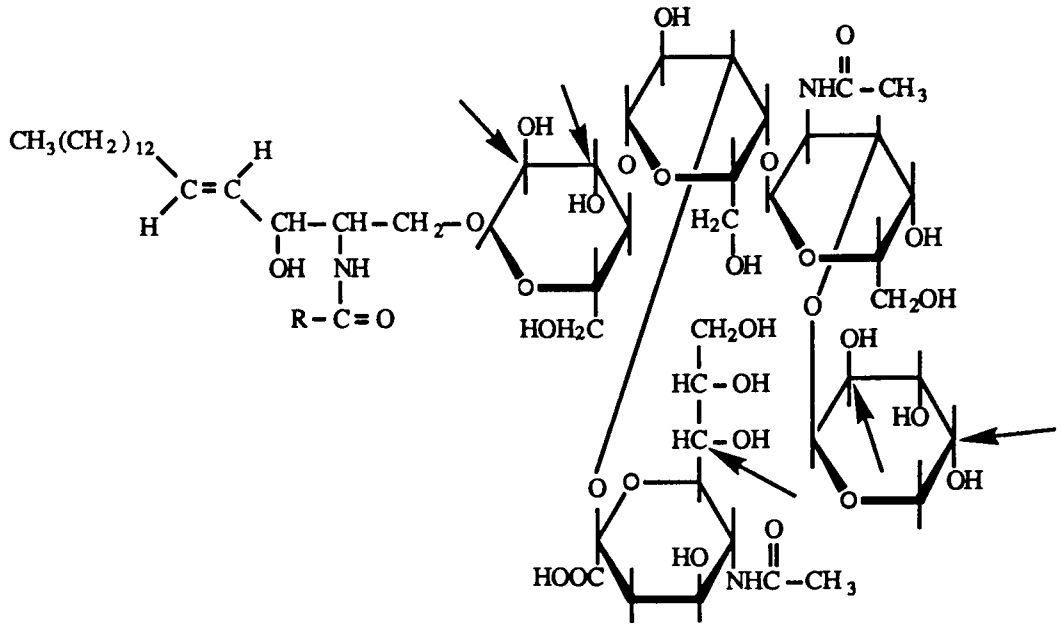

Figure 1-4. Structure of G_{M1}. Arrows indicate the potential positions of aldehyde generated by oxidation.

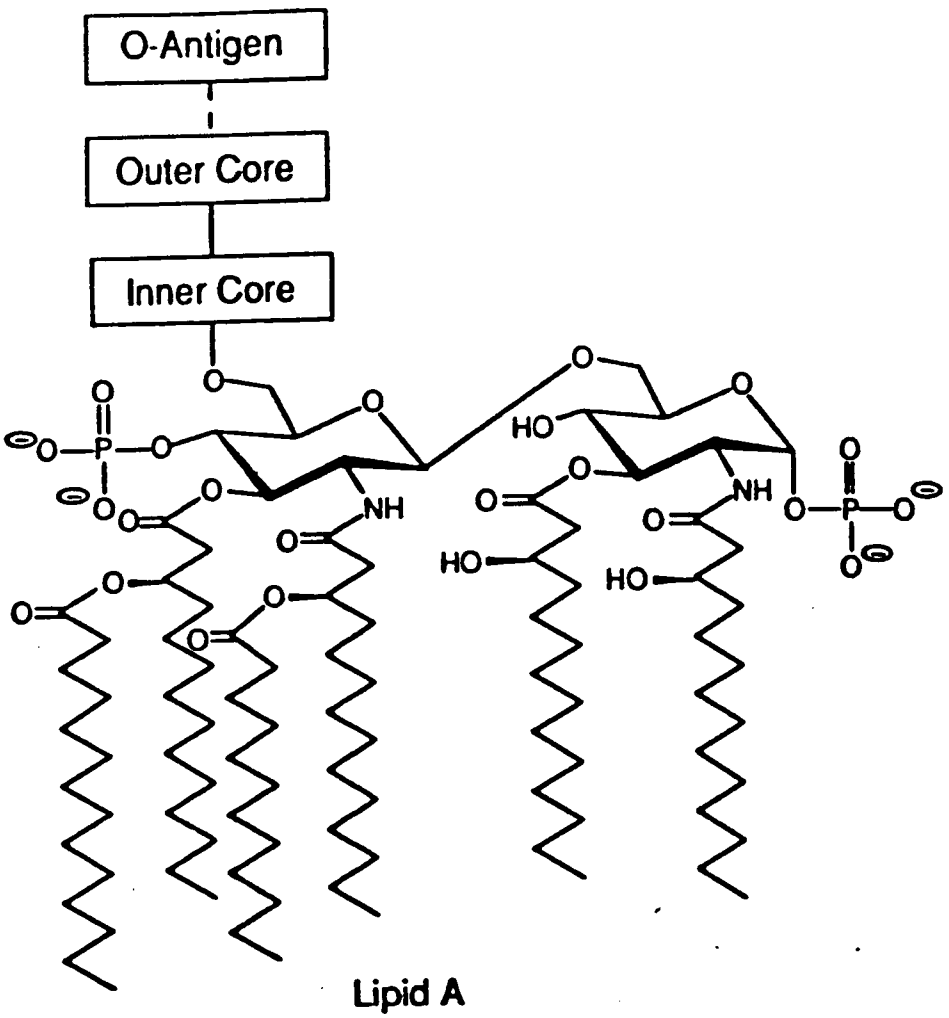

Figure 1-5. Structure of lipid A.

PART II

INTERACTION OF SYNTHETIC GLYCOPHOSPHOLIPIDS WITH PHOSPHOLIPID BILAYER MEMBRANES

1. Abstract

A series of glycophospholipids synthesized by coupling mono-, di-, or tri-saccharides to dioleoylphosphatidylethanolamine (DOPE) by reductive amination was used to investigate the interaction of glycophospholipids with phospholipid bilayer membranes. These synthetic glycophospholipids functioned as a stabilizer for the formation of DOPE bilayer vesicles. The minimal mol % of glycophospholipid needed to stabilize the DOPE vesicles were as follows: 8% N-neuraminlactosyl-DOPE (NANL-DOPE), 20% N-maltotriosyl-DOPE (MAT-DOPE), 30% N-lactosyl-DOPE (Lac-DOPE), and 42% N-galactosyl-DOPE (Gal-DOPE). The estimated hydration number of glycophospholipid in reverse micelles was 87, 73, 46, and 14 for NANL-DOPE, MAT-DOPE, Lac-DOPE, and Gal-DOPE, respectively. Thus, the hydration intensity of the glycophospholipid was directly related to the ability to stabilize the DOPE bilayer phase for vesicle formation. Glycophospho-lipids also reduced the transition temperature from gel to liquid crystalline phase (T_m) of dipalmitoylphosphatidylcholine (DPPC) bilayer. Interestingly, incorporation of NANL-DOPE induced a decrease of membrane fluidity of DPPC bilayer in the gel phase while other glycophospholipids had no effect. Also, low level of NANL-DOPE but not other glycophospholipids increased the transition temperature (T_H) from liquid crystalline to hexagonal phase of dielaidoylphosphatidylethanol-amine bilayers. These results showed that NANL-DOPE with a highly hydratable headgroup which provides a strong stabilization activity for the L_α phase of phospholipid membranes, may also be involved in specific interactions with neighboring phospholipids via its saccharide moiety.

2. Introduction

Glycolipids are involved in many biological processes including cellular recognition, cell surface receptors for toxins,drugs, natural agonists, and lectins, as well as immune events and others (for reviews, see refs 1-4). These functions are largely based on the saccharide structure of the glycolipids, but the ceremide moiety of the molecule also provides an unusual physical property for the cell membranes. The antigenesity of glycosphingolipids is dependent not only on the spatial configuration of the saccharide unit but on the ceremide portion of the lipid as well (5). Also, it has been shown that glycolipids cluster to form rigid domains in a bilayer membrane, possibly due to their extensive H-bonding capability (6) as well as the interdigitated hydrocarbon chains of the ceramide molecules (7). These rigid domains affect a number of important physical properties of the glycolipids including the conformation of the saccharide moiety (8), and the exit rate of the glycolipid in an exchange reaction (9,10). The potential biological significance of these properties has been proposed (11). The functional role of glycolipids as a structural element is also determined by the nature of the sugar headgroup (6).

In a structure-function study on the cryoprotection activity of glycolipids on phospholipid membranes, we have synthesized a series of phosphatidyl-ethanolamine derivatives by coupling mono-, di-, or tri-saccharides via reductive amination (12). The cryoprotective activity was correlated to the size of the saccharide moiety of the glycophospholipid with trisaccharide-PE conjugates exhibiting the highest activity. Interestingly, the monosacchride-PE conjugate not only lacks the cryoprotection activity, but also antagonizes the activity of trehalose, a well-established

cryoprotectant. Fourier transform infrared studies revealed that trehalose did not H-bond with the phosphate group of the monosaccharide-PE conjugates, which could be the basis of the antagonistic effect.

In the present study, the interactions of these synthetic glycophospholipid conjugates with phospholipid membranes have been systematically investigated. Stabilization of the bilayer structure of unsaturated PE by incorporation of glycophospholipid is demonstrated by vesicle formation and by the entrapment of a water-soluble dye in the vesicles. Perturbation of transition from the gel to the liquid-crystalline phase of DPPC bilayer by the glycophospholipids has also been studied by differential scanning calorimetry and fluorescence depolarization of diphenylhexatriene (DPH). The change of the hexagonal phase transition temperature of DEPE bilayer by the glycophospholipid has been examined by differential scanning calorimetry. The results of these studies should provide some insight to the interactions of glycolipids with phospholipid membranes.

3. Materials and methods

a. Materials

DOPE, DEPE, and DPPC were purchased from Avanti Polar Lipids Inc. (Birmingham, AL.). Calcein, galactose, lactose, maltotriose, and N-acetyl-neuramin-lactose were purchased from Sigma Chemical Co. Diphenyl-hexatriene (DPH) was purchased from Aldrich Chemical Company Inc. [^{3}H]H_2O was purchased from Amersham Co. (Arlington Height, Il.).

b. Synthesis of saccharide-DOPE conjugates

A series of saccharide-DOPE conjugates was synthesized by the reductive amination coupling method previously reported (12). Briefly, each saccharide (galactose, lactose, maltotriose, or N-acetylneuramin-lactose) was dissolved in 20 mM phosphate buffer (pH 7.5). The saccharide solution was mixed with DOPE dissolved in methanol. The molar ratio of sugar to lipid was 50:1 and the volume ratio of phosphate buffer to methanol was 1:10. An excess amount of $NaCNBH_3$ was added to the reaction mixture every 8 h during 48 h incubation at 40 °C with constant agitation. The reaction mixture was shaked with a mixture of water and chloroform. The saccharide-DOPE conjugates in the organic phase were harvested and further purified using preparative thin-layer chromatography on silica plates with the solvent system of chloroform:methanol:water (60:35:5). Purified conjugates run as single spots on high performance thin layer chromatography using the same solvent system. Purified conjugates were quantitated by the phosphate assay (13) and sugar assays (14). The ratios of phosphate to saccharide in all conjugates were approximately 1. All conjugates were also characterized by FTIR confirming the appearance of -OH streching and the disappearance of $-NH_2$ streching.

c. Vesicle preparation

Mixtures of varying amounts of glycophospholipid and DOPE were dried under N_2 stream and vaccum-desiccated for 30 min. PBS (pH 7.5) containing 50 mM calcein was added to the lipid mixture. The lipid suspension (5 mM in total lipid) was sonicated at room temperature using a

bath-type sonicator (Laboratory Supplies Inc., Hicksville, NY) to produce unilamellar vesicles. After 1 h incubation at room temperature, half of the vesicle preparation was utilized for 90° light scattering measurement at 660 nm to show vesicle formation using a Perkin Elmer LS5 spectrofluorometer. The rest was applied to a Bio-Gel A 0.5M column which was eluted with an isotonic PBS in order to remove the free unentrapped calcein. The recovered vesicles were then used for the fluorescence measurement to determine calcein entrapment. The fluorescence intensity of each sample was measured with the same spectrofluorometer (λ_{ex}=490 nm, λ_{em}=520 nm). The total fluorescence intensity of each sample was obtained by the addition of 50 µl deoxychholate (5 %) to lyse the vesicles. Percent fluorescence quenching of calcein entrapped in vesicles was calculated as:

$$\% \text{ quenching} = \frac{Ft - Fo}{Ft} \times 100$$

where Ft is the total fluorescence intensity and Fo is the observed fluorescence intensity of the vesicles.

d. Fluorescence anisotropy of diphenylhexatriene (DPH)

Lipid samples containing DPPC and a various amount of glycophospholipid were dried under N_2 stream and vacuum-desiccated for 30 min. All lipids (2 µ mole) were hydrated with 2 ml PBS (pH 7.5) at 45 °C for 10 min. Two µl of 2 mM DPH solution in tetrahydrofuran were added to the sample which was additionally incubated for 3 h at 45 °C and then cooled to room temperature. The fluorescence of DPH in each lipid suspension was measured using a Perkin -Elmer LS5 spectrofluorometer (λ_{ex}= 357 nm, λ_{em}= 430 nm) equipped with a pair of plain polarizers.

Fluorescence polarization was calculated using the following equation:

$$p = \frac{VV - \frac{VH \times HV}{HH}}{VV + \frac{VH \times HV}{HH}}$$

where VV, VH, HV, and HH are the fluorescence intensities measured at four different combinations of the polarizer position (V= vertical, H= horizontal). The fluorescence anisotropy was calculated as r = 2p/(3-p).

e. Differential scanning calorimetry

DPPC or DEPE samples containing various mol % of glycophospholipid were dried under N_2 stream and vacuum-desiccated for 30 min. Thirty μmole lipids were hydrated in 3 ml PBS (pH 7.5) at 45 °C for 4 h and then left overnight at room temperature for equilibration. Heating scans of the lipid samples were performed from 25 °C to 90 °C with a scan rate of 20 °C/h using a MC2 high sensitivity differential scanning calorimeter (Microcal Inc., Amherst, MA). T_m (transition temperature, L_β to L_α), T_H (transition temperature L_α to H_{II}) and enthalpy change were determined by a computer interfaced with the calorimeter.

f. Measurement of headgroup hydration in reverse micelles

A method for direct measurement of headgroup hydration of polar lipid in reverse micelles was developed by Sen and Hui (15). Briefly, 1 mg each glycophospholipid was dried under N_2 stream and vacuum-desiccated for 2 h. One ml pure dry hexane and 25 μl ^{3}H-labeled water (25 uCi/ml) were added to the dried lipid. The two phases were mixed vigorously using

a vortex mixer and then sonicated for 20 min in a bath type sonicator. After two phases were separated, an aliquot of the hexane phase was used for determination of radioactivity with a liquid scintillation counter. Another aliquot of the same hexane phase was used for phosphate assay (13). The number of water molecules associated per lipid molecule was calculated from the data.

g. Measurement of vesicle size

Lipid samples containing egg PC and glycophospholipids (4:1, molar ratio) were dried under N_2 stream and vacuum-desiccated for 30 min. Two hundred mM octylglucoside in PBS (pH 7.5) was added to the lipid suspension (10 mM) which was solutions were sonicated briefly until clarity and then dialyzed against PBS (pH 7.5). Vesicle size was estimated by quasi-elastic light scattering using a Coulter N4SD submicron particle analyzer (Coulter, Hialeah, FL.) and by negative-stain electron microscopy using 1 % uranium acetate as a stain (16).

4. Results

a. Stabilization of DOPE bilayer by glycophospholipid

Formation of stable DOPE bilayer vesicles by incorporating synthetic glycophospholipid was monitored by two different methods: 90° light scattering and fluorescence quenching of calcein entrapped in the vesicles. Formation of stable vesicles results in a low level of light scattering (17). Also, stable vesicles entrapping 50 mM calcein normally exihibit approximately 70% fluorescence quenching (18). In order to determine the

minimal mol % of synthetic glycophospholipid needed to stabilize the DOPE bilayer vesicles, various amounts of the glycophospholipid were incorporated into the lipid composition (Figure 2-1). The estimated minimal mol % of each glycophospholipid determined by the two methods was quite similar: 8% NANL-DOPE, 20% MAT-DOPE, 30% Lac-DOPE, and 42% Gal-DOPE. This pattern of the minimal mol % directly represented the order of stabilization activity of the glycophospholipid for the formation of the DOPE bilayer vesicles. Glycophospholipids with a larger saccharide moiety showed better stabilization activities. Among the glycophospholipids, the monosaccharide-DOPE (Gal-DOPE) had the poorest activity and the disaccharide-DOPE (Lac-DOPE) was the next. Moreover, NANL-DOPE was a better stabilizer than MAT-DOPE although both had trisaccharide headgroups. This may be due to the additional negative charge of the terminal sialic acid on the headgroup of NANL-DOPE.

b. Hydration number and vesicle curvature

Hydration intensity around the lipid headgroup and the hydration force in the interfacial region are major factors affecting lipid polymorphism (19). Recently, a method for direct measurement of headgroup hydration of phospholipid in reverse micelles has been developed (15). Hydration number of phospholipid measured by this method were in close agreement with those obtained from other measurements using phospholipids in the lamellar phase. As expected, hydration number of the synthetic glycophospholipid increased with the size of their sugar moieties (Table 2-I). For trisaccharide-DOPE's, the hydration number of NANL-DOPE was larger than that of MAT-DOPE, which must be due to the

negative charge of the terminal sialic acid in NANL-DOPE. The hydration number of Lac-DOPE was between those of MAT-DOPE and Gal-DOPE. As a control measurement, the hydration numbers of egg PC and DOPE were determined by the same method and found to be 25.3 and 9.3, respectively. These numbers are in close aggreement with those previously reported by others (20,21).

One of the major factors determining the vesicle curvature is the hydration force in the interfacial area of membrane (19). The hydration number measured above was clearly related to the vesicular curvature of the lipid bilayer containing glycophospholipid (Table 2-I). Vesicles prepared by the detergent dialysis method are at a relatively low energy state because minimal mechanical force is involved during the process. The size difference between vesicles prepared under the same conditions should reflect the difference in the intrinsic property of glycophospholipid affecting the vesicle curvature. Vesicle size was measured by two different methods: quasi elastic light scattering (QELS) and electron microscopy using negative staining (micrographs not shown). The size measurement of egg PC vesicles containing 25 mol % of glycophospholipid showed an inverse relationship to the hydration number of the glycophospholipid. Thus vesicles containing NANL-DOPE, the most hydratable glycophospholipid, had smallest mean diameter, whereas vesicles containing Gal-DOPE, the least hydratable glycophospholipid, had the largest mean diameter.

c. Effect of glycophospholipid on main phase transition and membrane fluidity of DPPC

Depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) is commonly used as a means of estimating membrane fluidity as well as revealing the phase transition from a gel to a liquid crystalline state (22,23). Fluorescence anisotropy of DPH is sensitive to the structural order of lipids in the membrane. The temperature dependence of fluorescence anisotropy of DPH was studied with DPPC bilayer vesicles containing various amount of glycophospholipid (Figure 2-2). Generally, addition of glycophospho-lipid resulted in a decrease in T_m and a broading of the transition of DPPC bilayer. Decrease in T_m by the addition of Gal-DOPE was larger than what was observed for NANL-DOPE at low mol % (Figure 2-2A). However, the addition of 40% NANL-DOPE seemed to abolish the clear phase transition or decreased it to below 25° C ,whereas an equal amount of Gal-DOPE reduced the T_m to about 30 °C (Figure 2-2B). The T_m change of DPPC bilayer by Lac-DOPE or MAT-DOPE was similar to what was observed for Gal-DOPE (data not shown).

Interestingly, addition of 5 mol % NANL-DOPE induced a reproducible increase of DPH anisotropy in the gel state of DPPC, whereas Gal-DOPE did not affect DPH anisotropy in the same state (Figure 2-2a). At 25 mol %, both glycophospholipids induced a reduction of the DPH anisotropy (Figure 2-2b). These results indicate that a relatively low mol % of NANL-DOPE, but not Gal-DOPE, has an ordering effect on DPPC bilayer in the gel phase. Lac-DOPE and MAT-DOPE showed almost the same effect as that of Gal-DOPE (data not shown). Addition of glycophospholipids did not affect the DPH fluorescence anisotropy of DPH in DPPC bilayer in the

liquid-crystalline state.

In order to examine such an unusual action of NANL-DOPE in DPPC bilayers in the gel phase, the same procedure was repeated with varying amounts of synthetic glycophospholipids at 25°C where the DPPC bilayer exists in the gel state (Figure 2-3). Addition of NANL-DOPE up to 15 % induced the fluidity decrease in DPPC bilayer. Lac-DOPE and MAT-DOPE induced a small decrease below 5 mol %. Steady decrease in anisotropy was observed for Gal-DOPE and DOPE at all molar ratios tested. These data suggest that the saccharide headgroups of glycophospholipids affect the acyl chain order of the DPPC bilayer membrane, which is sensed by the probe DPH. The fluidity increase of DPPC bilayer by 40 mol % NANL-DOPE was greater than what was observed for any other glycophospholipids at the same mol %.

d. DSC studies of the effect of glycophospholipids on main phase transition of DPPC

Differential scanning calorimetry (DSC) is a sensitive method to monitor changes of phase transition parameters (ΔT_m,ΔT_H, ΔH, and ΔS). Effects of NANL-DOPE and Gal-DOPE on the T_m of DPPC bilayer were compared to each other at the same mol % (Figure 4). Curve (A) in Figure 4 is a typical scan of pure DPPC bilayer, which shows a clear phase transition from gel (L_β) to liquid crystalline ($L\alpha$) at 42°C and a pretransition at 36°C. Incorporation of Gal-DOPE or NANL-DOPE into DPPC bilayers reduced the T_m of DPPC and abolished the pretransition. The ΔT_m induced by Gal-DOPE was greater than that by NANL-DOPE. These data are comparable with those of Figure 2-2. The peak of phase transition became

broader with an increase of the glycophospholipid content. Changes of enthalpy and entropy were also calculated from the DSC data. Table 2-II contains data for T_m, the enthalpy change, and the entropy change at each lipid composition. DPPC/NANL-DOPE bilayer showed larger changes of enthalpy and entropy during the phase transition than the pure DPPC bilayer. However, Gal-DOPE appeared not to affect the enthalpy and entropy of the DPPC bilayer although it effectively lowered the T_m.

e. Effect of glycophospholipid on hexagonal phase transition of DEPE

The DSC analysis was also used to study the effect of glycophospholipid on the phase transition from L_α to H_{II} of DEPE (Figure 2-5). It has been shown that a L_α phase-stabilizing amphiphilic molecule increases the hexagonal phase transition temperature (T_H) of DEPE, whereas a destabilizing one decreases the T_H (24, 25). Curve (A) in Figure 2-5 is a typical thermal scan of DEPE, showing a main transition (L_β -> L_α) at 37°C and a hexagonal phase transition (L_α -> H_{II}) at 66°C. Addition of 4 mol % NANL-DOPE induced an increase of the T_H of DEPE to 74°C and a decrease of the T_m down to 36.5°C. These data indicate that NANL-DOPE functions as an amphiphilic stabilizer for the L_α phase of DEPE bilayers. However, addition of 4% Gal-DOPE lowered the T_m to 35.5°C and also lowered the T_H to 63°C. This imdicates that Gal-DOPE funtions as a H_{II} phase stabilizer. The T_H change of DEPE upon adding various mol % of glycophospholipid is shown in Figure 2-5 insert. The T_H of DEPE increased with the mol % of NANL-DOPE, but decreased at all mol % of Gal-DOPE tested. Even though Gal-DOPE was a stabilizer to form DOPE bilayers (Figure 2-1), it did not function as a stabilizer for the Lα phase of DEPE bilayers. The hexagonal

phase transition of DEPE bilayer containing more than 6 mol % of Gal-DOPE was too broad to be recognized. Changes in the phase transition of DEPE bilayer by addition of Lac-DOPE or MAT-DOPE were quite similar to those observed with Gal-DOPE (data not shown).

5. Discussion

Glycolipids are structurally diverse and are major components of the cell membrane. While most studies on glycolipids are aimed at elucidating their functions as cell surface receptors, the physical properties of glycolipid in phospholipid membranes are not well established. In the present study we have systematically investigated the interaction of synthetic glycophospholipids with phospholipid bilayer membranes. For this investigation, mono-, di-, or tri-saccharides have been conjugated to DOPE, a common lipid anchor, by reductive amination.

These synthetic glycophospholipids differ from each other mainly in terms of the headgroup hydration (Table 2-I). The degree of hydration was directly proportional to the number of the neutral saccharide unit of the headgroup, with an increase of hydration number of approximately 30 H_2O molecules for each additional neutral saccharide unit. The presence of sialic acid in NANL-DOPE gave additional hydration numbers due to the negatively charged carboxyl group.

Due to the ability to increase the headgroup hydration, these glycophospholipids showed various activities to stabilize the DOPE bilayer vesicles. The activity was directly related to the hydration number of the glycophospholipid (Figure 2-1). Increased interfacial hydration would increase the repulsive force when vesicles are brought to close contact

during collision which is a major kinetic barrier for the destabilization of DOPE vesicles in aqueous suspension (26-28). Interfacial hydration is also a major factor in determining the curvature of bilayer at equilibrium (29). Highly hydrated bilayers may curve to a greater extent, hence forming smaller vesicles, than the poorly hydrated bilayers (Table 2-I).

We have chosen NANL-DOPE and Gal-DOPE for further detailed studies because they represent the extreme cases of hydration intensity of the glycophospholipids. Incorporation of both glycophospholipids into DPPC bilayer has brought the expected effect of decreasing the cooperativity of the L_β->L_α phase transition which was shown by both DPH fluorescence anisotropy (Figure 2-2) and DSC (Figure 2-4 and Table 2-II). Effects of broadening of the phase transition and decrease of the T_m were more pronounced with Gal-DOPE than NANL-DOPE (Figure 2-4 and Table II). The strong hydration intensity of NANL-DOPE may have partially stabilized the L_α phase of DPPC bilayer which is known to be more hydrated than the L_β phase (30). Addition of Gal-DOPE to DPPC bilayer should decrease the average hydration level of the bilayer as this glycophospholipid only binds with 14 H_2O molecules which are approximately half of the number for the PC molecule (15,20). These data have demonstrated the important role of interfacial hydration on the L_β->L_α phase transition.

Similar effect of hydration could also be observed on the influence of L_α->H_{II} phase transition of DEPE by the two glycophospholipids. This phase transition is highly sensitive to the incorporation of exogenous amphiphiles (24,25). Gal-DOPE, with which the hydration intensity is not much different from that of the PE itself (15,21), destabilized the L_α phase by lowering the T_H (Figure 2-5). On the other hand, the highly hydrated NANL-DOPE has brought a stabilization effect on the L_α phase by elevating

the T_H (Figure 2-5). Similar stabilization effect has been reported for amphiphiles possessing a charged headgroup which significantly increase the hydration intensity of the molecule (24,25).

Somewhat unexpected was the effect of NANL-DOPE on the gel (L_β) phase of DPPC (Figures 2-2, 2-3). A reproducible increase in the DPH fluorescence anisotropy was observed at temperatures below T_m for DPPC bilayers containing various amounts of NANL-DOPE, indicating that the lipids in the gel phase are more ordered due to the presence of this glycophospholipid. This is not likely an artifact of using DPH as a probe, because the transition enthalpy (ΔH), and entropy (ΔS) were also increased by about 15-18 % at low level NANL-DOPE incorporation as measured by DSC (Table 2-II). This phenomenon could be related to an earlier observation made by Tsao et al (31) who reported that ganglioside G_{D1a} at low mol % could enhance the ordering of PE bilayers. Since both NANL-DOPE and G_{D1a} contain sialic acid residues in their headgroups, specific interactions with the neighboring headgroups of phospholipids may bring about an enhanced interlipid interaction. Deuterium NMR and crystal structure studies have revealed strong intermolecular H-bondings which restricted the motion of the glycosyl headgroups of glycolipid in gel state membranes (32,33). Physical properties of glycolipid such as the acyl chain order and phase transition temperature can be attributed to the H-bonding network of the headgroups (34,35). The fluidity of the apolar interior of a bilayer decreases with increasing ganglioside content (34) and with a cooperative glycosyl headgroup interaction (35). The motion of a glycolipid headgroup is much slower than that of a phospholipid headgroup and the reduced mobility of the saccharide group may thus affect the mobility of the acyl chain (36-38).

In summary, this study has demonstrated the role of saccharide headgroups of glycolipids in phospholipid polymorphism. We believe that most of the observed effects can be attributed to the hydration intensity of the saccharide headgroup. However, the negatively charged sialic acid residue of a glycolipid may influence the physical properties of phospholipid by more than just the high level of headgroup hydration. Specific interactions such as H-bondings with the neighboring phospholipids should also be taken into consideration. This hypothesis will be tested in future studies.

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Appendix

Table 2-I

Hydration number and effect of glycophospholipid on liposome size.

Lipid	Hydration Number[1]	Liposome Diameter (nm)[2]	
		QELS[3]	E.M.[4]
None	-	196 (47)	210 (65)
Cholesterol	2.5 (0.3)	292 (60)	310 (45)
Gal-DOPE	14.4 (2.5)	164 (38)	196 (35)
Lac-DOPE	46.5 (6.7)	138 (46)	159 (21)
MAT-DOPE	73.1 (8.8)	124 (38)	141 (60)
NANL-DOPE	87.0 (9.1)	75 (22)	46 (8)

[1]Hydration number of glycophospholipid was measured in the reverse micelle phase. Mean (S.D.), n=3.

[2]Liposomes composed of egg PC:glycophospholipid or cholesterol (3:1, molar ratio) prepared by a dialysis method (detailed in the method section).

[3]Measured with a Coulter N4SD submicron particle analyzer.

[4]Measured by electron microscopy using 1 % uranium acetate as negative stain. At leat 100 vesicles were measured in each preparation. Mean (S. D.).

Table 2-II

Effect of glycophospholipid on the phase transition of DPPC bilayer

Glycophospholipid[1]		T_m (°C)	ΔH(kcal/mol)	ΔS(cal/mol·deg)
None		41.7	8.7	27.8
Gal-DOPE	2 %	40.9	9.1	29.0
	4 %	40.1	8.8	28.1
	6 %	39.1	7.9	24.3
NANL-DOPE	2 %	41.3	10.1	32.3
	4 %	40.9	10.4	33.1
	6 %	39.7	9.9	31.5

[1]DPPC containing various amount of glycophospholipid was used for differential scanning calorimetry measurements as described in Materials and Methods.

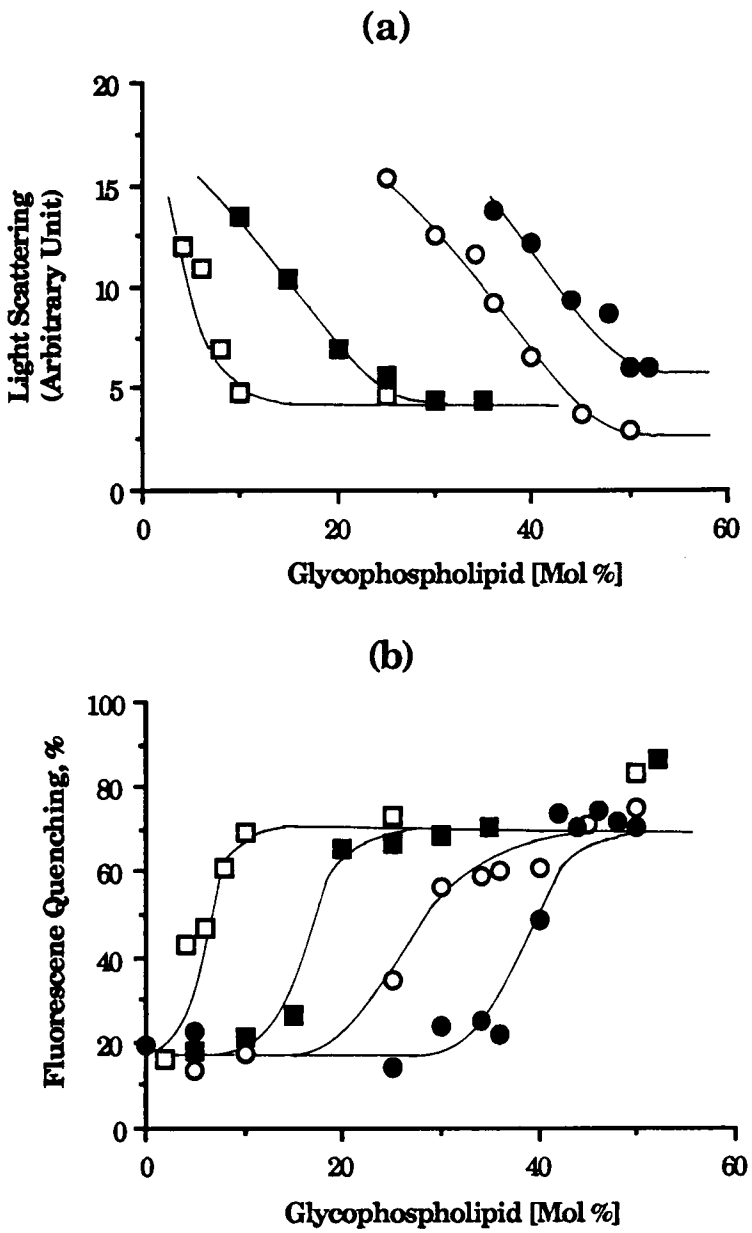

Figure 2-1. DOPE bilayer stabilization by synthetic glycophospholipids. (a) 90° light scattering of the sonicated liposomes containing various amount of Gal-DOPE (●), Lac-DOPE (○), MAT-DOPE (■), or NANL-DOPE (❑). (b) Percent fluoroscence quenching of calcein entrapped in the same liposomes.

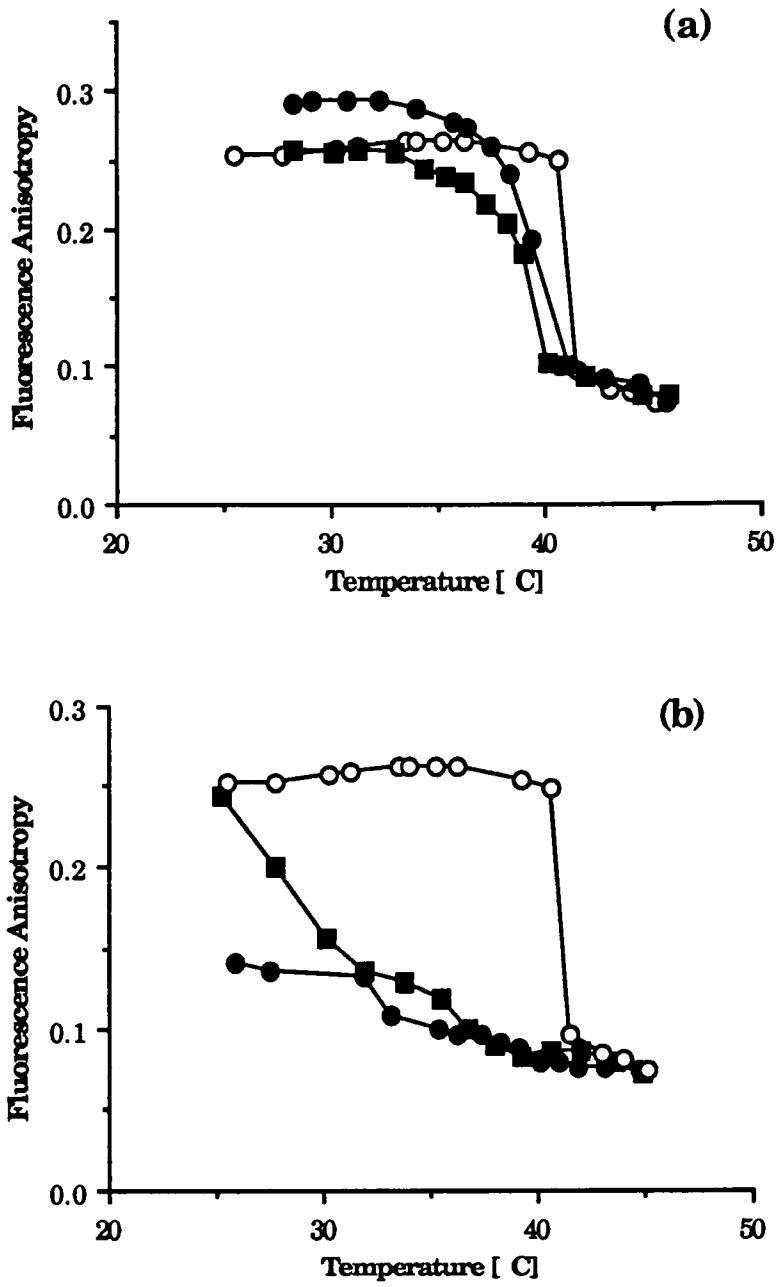

Figure 2-2. Fluorescence anisotropy of DPH in DPPC bilayer containing various glycophospholipids. Fluorescence anisotropy of DPH in DPPC bilayer containing none (○), Gal-DOPE (■), or NANL-DOPE (●) was measured at various temperature. Graph (a), 5 mol % glycophospholipids. Graph (b), 40 mol % glycophospholipids.

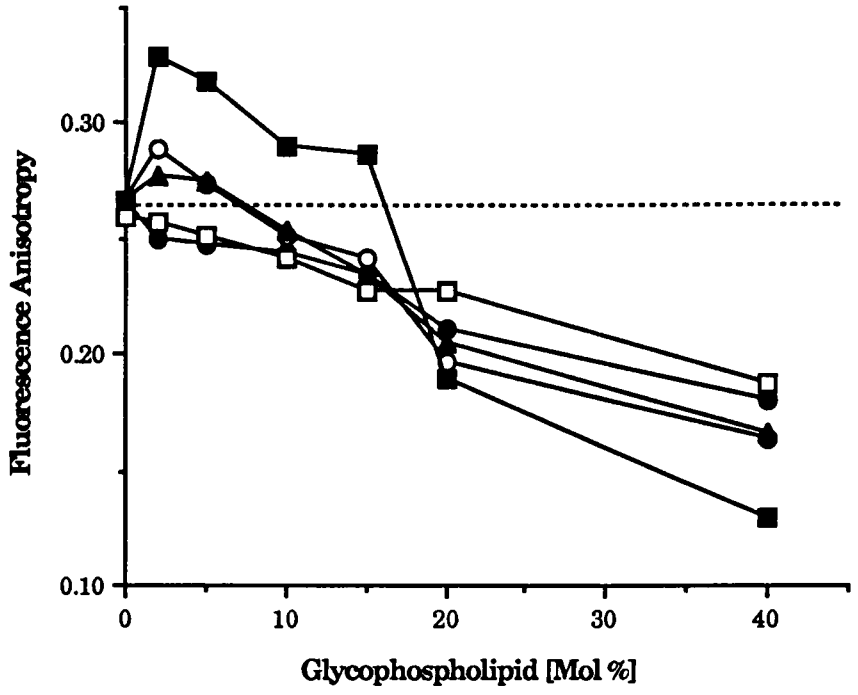

Figure 2-3. Fluorescence anisotropy of DPH in DPPC bilayer containing various glycophospholipids in the gel phase. Fluorescence anisotropy of DPH in DPPC bilayer containing Gal-DOPE (●), Lac-DOPE (▲), MAT-DOPE (○), NANL-DOPE (■) or DOPE (❑) was measured at 25°C. The horizontal dotted line represents the anisotropy for the pure DPPC bilayer.

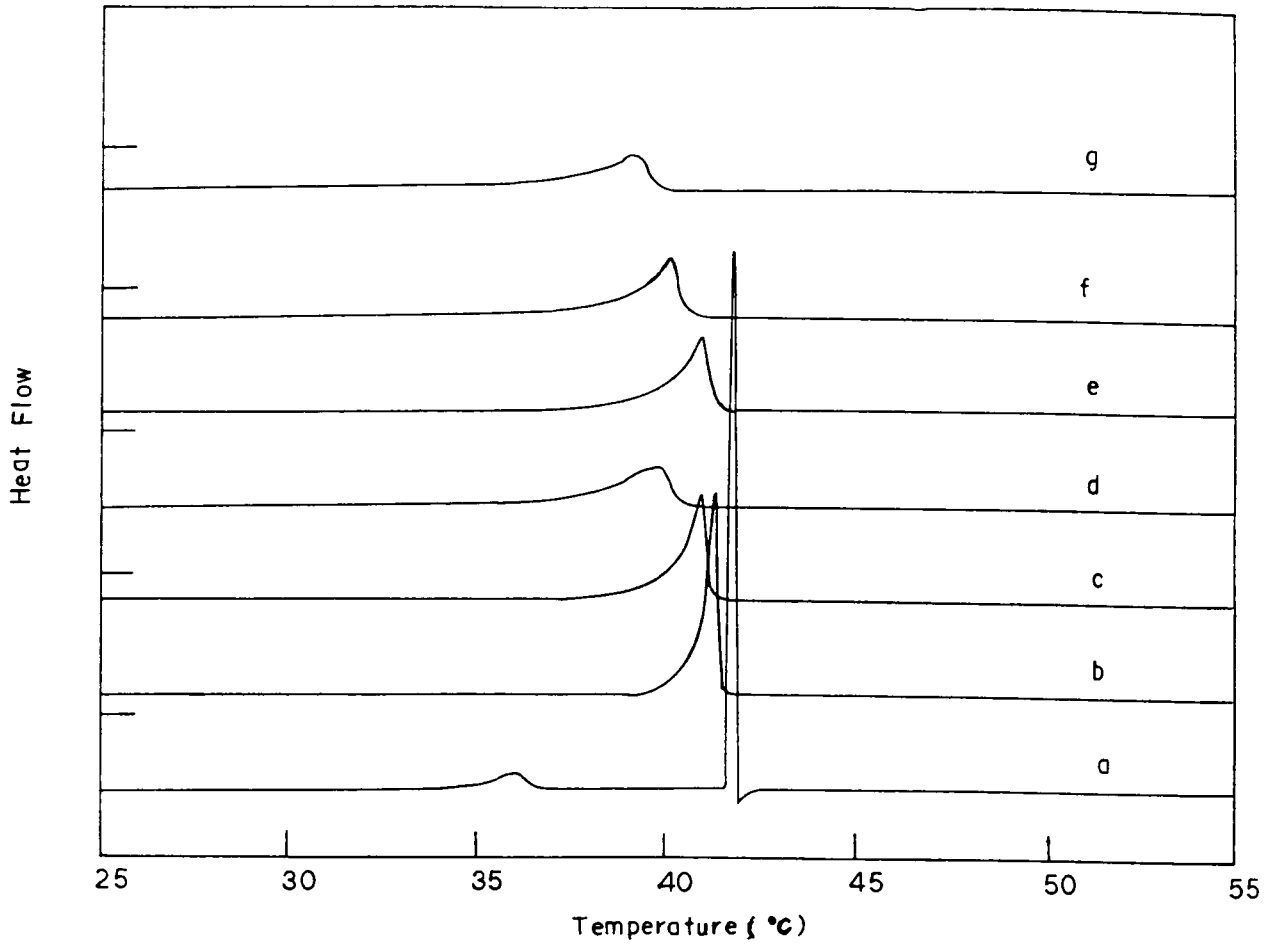

Figure 2-4. Effect of glycophospholipid on the DSC scan of DPPC bilayer. Scans of DPPC bilayer samples containing (a) none, (b) 2 % NANL-DOPE, (c) 4 % NANL-DOPE, (d) 6 % NANL-DOPE, (e) 2 % Gal-DOPE, (f) 4 % Gal-DOPE, or (g) 6 % Gal-DOPE are shown.

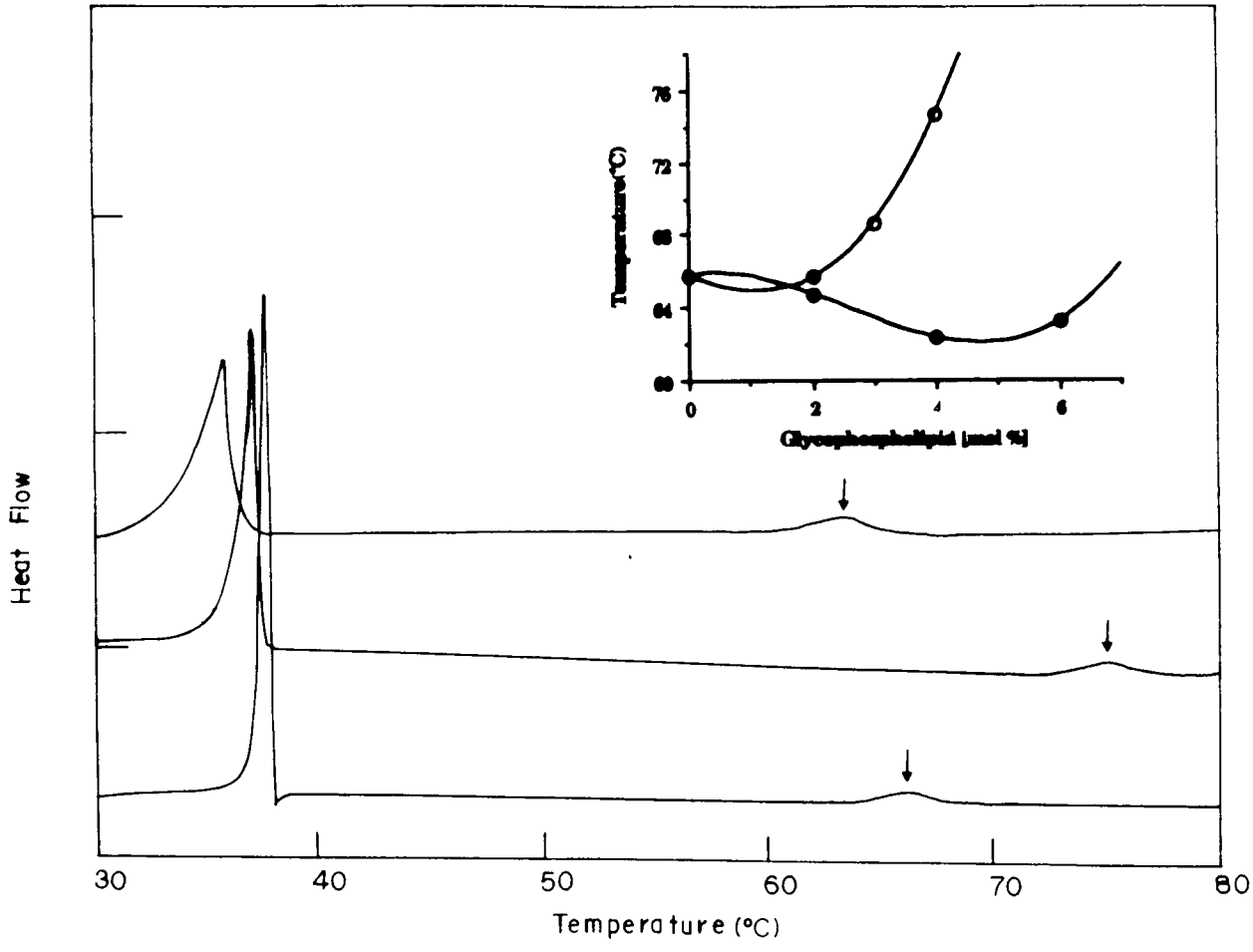

Figure 2-5. Effect of glycophospholipid on the DSC scan of DEPE bilayers. Scan of DEPE bilayer containing (a) none, (b) 4 % NANL-DOPE, or (c) 4 % Gal-DOPE are shown. The peak temperature of the hexagonal phase transition is marked with arrow. Insert figure shows the effect of glycophospholipid on the T_H of DEPE bilayer containing various amount of Gal-DOPE (●) or NANL-DOPE (○) measured by DSC.

PART III

CRYOPROECTIVE ACTIVITY OF SYNTHEIC GLYCOPHOSPHOLIPIDS AND THEIR INTERACTIONS WITH TREHALOSE

1. Abstract

A series of glycophospholipids have been synthesized by conjugating mono-, di-, and tri-saccharides with dioleoylphosphatidylethanolamine (DOPE) by reductive amination. These synthetic glycophospholipids were incorporated into egg phosphatidylcholine (PC) vesicles and tested for the release of vesicle-entrapped calcein after 5 cycles of freeze and thaw. The ability to protect the vesicles against freeze-thaw damage increases with the number of saccharide unit of the glycophospholipids. N-isomaltotriosyl-DOPE and N-(N-acetylneuramin-lactosyl)-DOPE had approximately equal activity in cryoprotection; whereas N-lactosyl-DOPE and N-galactosyl-DOPE were without effect. However, the cryoprotective effect of trehalose, a well-known cryoprotectant for cells and liposomes, was significantly reduced with the incorporation of N-lactosyl-DOPE and N-galactosyl-DOPE, but not with the trisaccharide-DOPE conjugates. Thus, the mono-and di-saccharide-DOPE conjugates are anticryoprotectants. FTIR studies of mixtures of trehalose and glycophospholipid revealed that trehalose forms H-bonding with the phosphodiester group of N-(N-acetylneuramin-lactosyl)-DOPE but not that of N- galactosyl-DOPE. The presence of interaction of trehalose with the trisaccharide-DOPE conjugate and the absence of interaction with the monosaccharide-DOPE conjugate were also demonstrated by the change of the H_{II} phase transition temperature of dielaidoyl phosphatidylethanolamine as measured by the differential scanning calorimetry. The ability of trehalose to restore the membrane order of the rigid dipalmitoyl phosphatidylcholine bilayer perturbed by the presence of glycophospholipids is also correlated with the ability of trehalose to interact with trisaccharide-DOPE conjugate but not with the

monosaccharide-DOPE conjugate. These observations have shed some light on the mechanism of the cryoprotective activity of the membrane bound carbohydrates.

2. Introduction

Stability of the biological membrane against freeze-thaw or dehydration conditions is of vital importance for the survival of the organism in the extreme environment. Phase transition and/or phase separation of the membrane lipids under the dehydration conditions causes the breakdown of the membrane permeability barrier (1,2). Saccharide molecules, such as trehalose, can protect the membrane from dehydration damage, and have served as a natural cryoprotectant for a number of organisms (1,3). The mechanism of the cryoprotective action of trehalose has been related to its ability to form hydrogen bondings with the headgroup of phospholipids (4,5) and to reduce the L_β-L_α phase transition temperature of the membrane lipids (5,6). Thus, the membrane lipids are still in the L_α phase when dehydrated in the presence of trehalose; whereas the lipids are in the L_β phase when dehydrated in the absence of the cryoprotectants (7).

Cryostability is also important for liposomes and other artificial lipid assemblies. Unfortunately, most liposome compositions are not stable under the cryopreservation conditions; only a few preparations have been reported as having a required stability (8-11). An effective approach to improve the cryostability has been taken by incorporating synthetic glycolipids in the liposome membrane to produce a local high concentration of the cryoprotective saccharide at the liposome surface.

Thus, galactose and maltose residues have been linked to cholesterol via a polyoxyethylene spacer arm and the resulting glycolipid exhibits a cryoprotective activity when incorporated into the liposome membrane (12,13). This promising approach suggests that the surface carbohydrate moieties can participate in H-bonding with the headgroups of the neighboring phospholipids (5,6,14,15), mimicking the effect of bulk trehalose at high concentrations.

We have undertaken a systematic study to examine the effect of saccharide structure on the cryoprotective activity of the glycolipids. Our approach is to conjugate mono-, di- and tri-saccharides of defined structure and purity to a common phospholipid structure and study their cryoprotective activities. Furthermore, the cryostability of the lipid vesicles containing synthetic glycophospholipids has been examined in the presence of trehalose to see if the surface saccharide residues interact with the bulk saccharide to produce a synergistic activity in cryoprotection. A number of unexpected observations have been made which should shed light on the mechanism of action of the saccharide cryoprotectants.

3. Materials and Methods

a. Materials

DOPE, DEPE, DPPC, and egg PC were purchased from Avanti Polar Lipids, Inc. Calcein, galactose, lactose, isomaltotriose, N-acetylneuramin-lactose, and trehalose were purchased from Sigma Chemical Co. DPH was purchased from Aldrich Chemical Company,Inc.

b. Synthesis of saccharide-DOPE conjugates

Saccharide-DOPE conjugates were synthesized by reductive amination with cyanoborohydride (16,17). Saccharides (galactose, lactose, isomaltotriose, or N-acetylneuramin-lactose) were dissolved in 20 mM phosphate buffer, pH 7.5. The saccharide solution was mixed with DOPE dissolved in CH_3OH. The molar ratio of sugar to lipid was 5:1 and the volume ratio of phosphate buffer to methanol was 1:10. 20 mg of $NaBH_3CN$ was added to the saccharide-lipid mixture every 8 h during the incubation for 48 h at 37 °C with shaking. The reaction mixture was separated into two layers by phase separation using water and chloroform. The saccharide-lipid conjugates in the organic layer were purified using the preparative thin layer chromatography on silica plates with the solvent system of chloroform:methanol:water = 60:35:5. The purified conjugates run as a single spot on the thin layer chromatography using the same solvent system. The R_f values of the conjugates were: 0.53, 0.41, 0.27, 0.26 for Gal-DOPE, Lac-DOPE, IMT-DOPE and NANL-DOPE, respectively. The purified conjugates were quantitated by the phosphate assay (18) and sugar assays (19,20). Anthrone assay was done for Lac-DOPE and IMT-DOPE and resorcinol assay was done for NANL-DOPE. The sugar moiety of Gal-DOPE was not detectable by the anthrone assay because the amide-bonded galactose was not hydrolysable by anthrone reagent. The ratios of lipid to sugar of Lac-DOPE, IMT-DOPE and NANL-DOPE were 0.98, 0.94, and 1.07 respectively. All conjugates were also characterized by FTIR confirming the appearance of -OH stretching and the disappearance of $-NH_2$ bending (data not shown).

c. Liposome preparation

Lipid mixtures of varying amounts of sugar-DOPE and egg PC were dried under N_2 stream and vacuum-desiccated for 30 min. The lipids (5 mM) were hydrated in PBS containing 50 mM calcein and 100 mM trehalose or PBS containing only 50 mM calcein. They were sonicated at room temperature using a bath-type sonicator (Laboratory Supplies, Inc., Hicksville, NY) to form unilamellar vesicles. After 1 h at room temperature, free untrapped calcein was removed from the liposomes by using a Bio-Gel A0.5M column eluted with an isotonic PBS. The diameter of the liposomes was measured by the laser light scattering using a Coulter N4SD.

Liposome samples were diluted to 2 mM in 0.3 ml with an isotonic PBS with or without 100 mM trehalose. Samples were submerged into a dry ice/methanol bath for freezing for 3 min. and thawed at room temperature. Each freeze-thaw cycle took approximately 40 min and a total of 5 cycles was done for each sample.

d. Test of liposome stability against freeze-thaw damage

The fluorescence intensity of each sample was measured with a Perkin-Elmer LS-5 spectrofluorometer (λ_{ex} = 490 nm, λ_{em} = 520 nm). The total fluorescence intensity was obtained by the addition of 50 µl of 5 % deoxycholate to lyse the liposomes. Percent lysis of liposomes was calculated with the equation:

$$\% \text{ lysis} = \frac{F_t F_o' - F_o F_t'}{F_t' F_t - F_t' F_o} \times 100$$

where F_o is the fluorescence intensity of liposomes and F_t is the total fluorescence intensity before freeze-thawing. F_o' and F_t' are the fluorescence intensity of liposome and total fluorescence intensity after freeze-thawing, respectively.

e. Fourier transform infrared spectroscopy

FTIR spectra of saccharide-DOPE and saccharide-DOPE/trehalose mixture (mole ratio 1:5) were recorded using a Bio-Rad FTS-7 spectrometer assisted data station. The samples were prepared by lyophilizing a liposome suspension (2 μmole lipid) with or without trehalose. The lyophilized sample was mixed with 100 mg dry KBr and was ground to a fine powder using a mortar and pestle, pressed into a disc, and mounted into the sample holder. The spectra were recorded as absorbance in the region of the phosphate asymmetric stretching (1200 - 1300 cm^{-1}).

f. Differential scanning calorimetry

DEPE samples containing saccharide-DOPE (4 mol %) were dried under a N_2 stream and vacuum-desiccated for 30 min. The lipids (10 mM) were hydrated in PBS or PBS containing 0.1M trehalose at 45 °C for 4 h and then left overnight at room temperature for equilibration. Heating scans of the lipid samples were done between 25 °C and 90 °C with a scan rate of 20 °C/h using a MC-2 high sensitivity differential scanning calorimeter (Microcal Inc., Amherst, MA). T_H was the peak temperature of the hexagonal phase transition determined by a computer.

g. Fluorescence anisotropy of DPH

Lipid samples containing DPPC and saccharide-DOPE (40 mol %) were dried under a N_2 stream and vacuum-desiccated for 30 min. All samples were hydrated with PBS (pH 7.5) at approximately 45 °C. An aliquot of 2 μl of a 2 mM DPH solution in tetrahydrofuran was added to the sample followed by a 10-min period of incubating and vortexing at approximately 45 °C. Samples were additionally incubated for 3 h at 45 °C before used for the fluorescence polarization measurement. Fluorescence of DPH was measured using a Perkin-Elmer LS-5 spectrofluorometer (λ_{ex} = 357 nm, λ_{em} = 430 nm) equipped with a pair of plane polarizers. Fluorescence polarization was calculated using the following equation:

$$P = \frac{VV - \frac{VH - HV}{HH}}{VV + \frac{VH - HV}{HH}}$$

where VV, VH, HV and HH are the fluorescence intensities measured at four different combinations of the polarizer position. The fluorescence anisotropy r is calculated as r = 2P/(3-P).

4. Results

a. Cryoprotective Effect of Glycophospholipids

We have synthesized four glycophospholipid conjugates which differ only in the saccharide structure. Gal-DOPE contains a gal residue directly linked to the NH_2 group of DOPE. Lac-DOPE contains a disaccharide unit, i.e. lactose, linked to DOPE. IMT-DOPE and NANL-DOPE contain a

trisaccharide unit, isomaltotriose and N-acetylneuramin-lactose, on the same DOPE structure, respectively. These lipids were mixed with egg PC and used to prepare small unilamellar vesicles entraping a fluorescence marker, calcein. The fluorescence of calcein is self-quenched at high concentrations (21). For example, approximately 80% quenching was observed for vesicles containing 50 mM entrapped calcein (22). As calcein is released from the vesicles as a result of the freeze-thaw damage, fluorescence quenching decreases with increasing cycles of freeze and thaw. We have used 5 cycles of freeze-thaw to test the cryostability of the vesicles.

Vesicles containing various amounts of glycophospholipid were tested in the presence or absence of 100 mM trehalose in PBS, pH 7.4. The results shown in Figure 3-1 clearly demonstrate that the vesicles containing trisaccharide-conjugated DOPE had a significant cryostability against freeze and thaw (Figure 3-1c,d). The cryoprotectant effect of both trisaccharide-DOPE conjugates were very similar and increased with increasing concentration of the glycophospholipid. For example, vesicles containing 75% of either IMT-DOPE or NANL-DOPE showed approximately 40% dye release after 5 cycles of freeze and thaw. Since NANL-DOPE contains a negatively charged N-acetyl neuraminyl group and IMT-DOPE does not, it appears that the cryoprotectant activity of the trisaccharide-DOPE conjugates does not depend on the charge content of the saccharide group. Mono- and di-saccharide-DOPE were without any cryoprotective activity up to 75% concentration. Vesicles containing these glycophospholipids showed approximately 80% release of calcein which is the same as the pure egg PC vesicles (Figure 3-1a,b). Thus, the cryoprotective effect is limited to the trisaccharide-DOPE conjugates;

conjugates with a smaller saccharide unit are without any effect.

Addition of 100 mM trehalose to vesicles during the freeze-thaw cycles had reduced the release of calcein from the pure egg PC vesicles to about 17%, indicating the cryoprotective effect of this disaccharide (Figure 1). However, it is clear from Figure 3-1a and b that Gal-DOPE and Lac-DOPE had an antagonizing effect on trehalose. The stability of the vesicles progressively decreased with increasing concentration of the mono- and disaccharide-DOPE. The antagonistic effects of these two glycophospholipids were approximately the same. For example, vesicles containing 75% Gal-DOPE showed approximately 50% calcein release after 5 cycles of freeze-thaw in the presence of trehalose; whereas vesicles containing the same concentration of Lac-DOPE showed approximately 40% dye release under the same conditions (Figure 3-1a,b). Thus, glycophospholipids containing one or two saccharide residues are "anticryoprotectants", i.e. they do not themselves show any cryoprotection effect, yet they antagonize the cryoprotective effect of trehalose. Glycophospholipids containing trisaccharide showed no interference on the trehalose effect (Figure 3-1c,d). In the case of IMT-DOPE, a small synergistic effect of the glycophospholipid with trehalose was seen (Figure 3-1c).

To test if the DOPE moiety of the glycophospholipid conjugates is responsible for their cryoprotective and anticryoprotective activities described above, we have examined the effect of DOPE on the vesicle stability against the freeze-thaw damage. It is clear from the data in Figure 2 that DOPE up to 75% in egg PC had no cryoprotective activity; vesicles containing various amounts of DOPE showed the same level of lysis as the ones without DOPE. Furthermore, DOPE up to 50% did not

interfere with the ability of trehalose to protect the vesicles against the freeze-thaw damage. Vesicles containing 75% DOPE showed some lysis in the presence of trehalose. This is because these vesicles were unstable even in the absence of freeze and thaw cycles, probably due to the high tendency of the vesicle lipids to undergo the bilayer-to-H_{II} phase transition (23). Thus, we conclude that DOPE itself does not exhibit any cryoprotective nor anticryoprotective activities.

When the liposome stability was tested in the presence of trehalose, we had noticed that the cryoprotective effect of trehalose was the same no matter if there was an equimolar amount of trehalose inside the liposomes or not (data not shown). This was the case for liposomes containing various amounts of glycophospholipid conjugates. These results indicate that the cryoprotective effect of trehalose is acting on the outer surface of the liposome membrane.

b. Anticryoprotection Effect of Gal-DOPE

The anticryoprotection activity of glycophospholipid was further investigated by using Gal-DOPE. It was possible to prepare vesicles with pure Gal-DOPE and calcein could be entrapped in the vesicles. We have thus compared the stability of the vesicles composed of pure Gal-DOPE with those composed of pure egg PC as a function of increasing trehalose concentration (Figure 3-3). For egg PC vesicles, 10 mM trehalose had shown a nearly complete protection of the calcein release against 5 cycles of freeze-thaw; whereas it required 1 M trehalose to show the same complete protective effect for the Gal-DOPE vesicles. Such 100-fold difference in the sensitivity to trehalose indicates that the presence of

monosaccharide residues on the phospholipid head group has significantly modified the interaction of trehalose with the phospholipid. It has been shown that the phospholipid head group greatly influences the degree of trehalose protection while fatty acyl chain composition is considerably less of importance (11).

c. Interactions of trehalose with glycophospholipids studied by FTIR

To examine the possibility of a direct interaction between trehalose and the glycophospholipids, we have measured the FTIR spectra of a dehydrated mixture of trehalose and NANL-DOPE or trehalose and Gal-DOPE (Figure 3-4). It has been shown that the P-O asymmetric stretching band of the phospholipid is shifted to a lower waver number if the phosphodiester group of the phospholipid is H-bonded (24). As can be seen in Figure 3-4, this was the case for the NANL-DOPE as compared to Gal-DOPE. The peak position of the P-O asymmetric stretching band was shifted from 1240 cm^{-1} in Gal-DOPE to 1235 cm^{-1} in NANL-DOPE, indicating that phosphodiester group of NANL-DOPE was involved in H-bonding. Spectra in Figure 4 also demonstrates that in the presence of trehalose the P-O asymmetric stretching band was further shifted (1235 to 1230 cm^{-1}). In contrast, no shift in the peak position was observed when Gal- DOPE was mixed with the same amount of trehalose; the peak stayed at 1240 cm^{-1} (Figure 3-4b). This result demonstrates that the cryoprotectant glycophospholipid (NANL-DOPE), but not the anticryoprotectant glycophospholipid (Gal-DOPE), can form H-bonding with trehalose at its phosphodiester group.

d. Effect of glycophospholipids and trehalose on the H_{II} phase transition of DEPE

The $L\alpha$–H_{II} phase transition of DEPE is very sensitive to the presence of another amphiphile (25). In general, amphiphiles which increase the interfacial hydration stabilize the L_α phase and increase the H_{II} phase transition temperature (T_H) and vice versa (25-27). We have examined the effect of saccharide-DOPE conjugates on the H_{II} phase transition of DEPE (Figure 3-5). Four mol % of Gal-DOPE had decreased T_H of DEPE from 66 °C to 63 °C (compare traces a and c), whereas NANL-DOPE at three mol % had increased the T_H to 69 °C (trace e). This result indicates that the trisaccharide-DOPE conjugate increases the interfacial hydration and stabilizes the L_α phase of DEPE. The monosaccharide-DOPE showed the opposite effect. Trehalose (0.1 M) alone also decreased T_H to 64 °C (trace b), in agreement with the previous reports (28). Trehalose and Gal-DOPE showed a synergistic effect by further decreasing T_H to 59 °C when both were added to DEPE (compare traces c and d). Trehalose was also antagonistic on the effect of NANL-DOPE; it had brought the T_H to 62.6 °C when both were added to DEPE (compare traces e and f). These results indicate that Gal-DOPE decreases the interfacial hydration of the DEPE bilayer and the effect is additive to the dehydration effect of trehalose. On the other hand, trehalose showed a dominating effect on NANL-DOPE by completely abolishing the hydration effect of this glycophospholipid. The latter effect is probably manifested as the result of direct interaction between trehalose and NANL-DOPE as shown by the data of FTIR studies (Figure 3-4).

e. Bilayer disordering effect of glycophospholipids measured by the fluorescence anisotropy of DPH

We have incorporated NANL-DOPE or Gal-DOPE into DPPC bilayer and studied the bilayer disordering effect by using a hydrophobic fluorescence probe DPH at a temperature (33 °C) below the chain-melting temperature of pure DPPC (41.5 °C). As can be seen in Table 3-I, both NANL-DOPE and Gal-DOPE showed a strong disordering effect on the DPPC bilayer. The fluorescence anisotropy had decreased from 0.266 for pure DPPC to 0.124 and 0.136 for DPPC containing 40 mol % of NANL-DOPE and Gal-DOPE, respectively. However, trehalose showed an ordering effect on the NANL-DOPE containing bilayer by increasing the anisotropy value to 0.157; whereas it had no significant effect on the pure DPPC bilayer or bilayer containing Gal-DOPE. These results demonstrate that trehalose can directly interact with NANL-DOPE, but not with Gal-DOPE, to partially restore the bilayer rigidity of DPPC at a temperature below the chain-melting temperature of DPPC.

5. Discussion

Surface bound carbohydrates are effective in the protection of the liposomal membrane from the damage of freeze and thaw or freeze and dry (12,13). In the present study, we systematically investigate the saccharide structure of the membrane bound carbohydrates responsible for the cryoprotectant activity. Mono-, di- and tri-saccharides of different structures have been conjugated to a common lipid anchor, i.e. DOPE, by a reductive amination reaction. The saccharide-DOPE conjugates are then incorporated into the lipid vesicles for the testing of cryoprotective activity.

It is shown by the data in Figure 1 that trisaccharide-DOPE conjugates showed some cryoprotectant activity, but the di- and monosaccharide-DOPE conjugates did not. It has been proposed that the cryoprotective activity of free saccharide depends on the H-bonding interaction of the saccharide with the headgroups of the phospholipids (4,5,7). A saccharide-DOPE conjugate with an extended and flexible saccharide chain such as the trisaccharides can conceivably H-bond with the neighboring phospholipid headgroups; whereas the conjugates with shorter saccharide chain can not easily do so. The terminal galactose residue of Gal-DOPE loses the rigid cyclic structure after the reductive amination reaction. The disaccharide moiety of Lac-DOPE essentially functions as a monosaccharide linked to DOPE via a polyol spacer arm. Meanwhile, the trisaccharide moieties of IMT-DOPE and NANL-DOPE function as a cryoprotectant disaccharides with extended and flexible polyol spacer arms, which essentially act as free disaccharides at the membrane surface. It is well known that disaccharides are very effective cryoprotectants while monosaccharides are ineffective (1,28). Trisaccharide-DOPE conjugates may also increase the interfacial hydration when the membranes are dehydrated during the freeze-thaw cycles, thus protecting the membranes from damage.

This hypothesis is supported by the data in Figure 5 The value of the H_{II} phase transition temperature of DEPE is a sensitive parameter of the membrane hydration (29). NANL-DOPE efficiently increased the T_H of DEPE, whereas Gal-DOPE decreased the T_H. This result indicates that NANL-DOPE increases the interfacial hydration and Gal-DOPE has an opposite effect. However, this mechanism is not supported by the observation that NANL-DOPE and IMT-DOPE had a similar cryoprotective

effect (Figure 3-1c,d). One would expect that NANL-DOPE, being negatively charged in the saccharide group, has a stronger activity in increasing the interfacial hydration than the IMT-DOPE which contains an uncharged saccharide group. The third possible mechanism of the cryoprotection effect of the saccharide-DOPE conjugates relates to the hypothesis that freezing damage of the membrane is a result of membrane contact and fusion during the dehydration process (30,31). Trisaccharide-DOPE conjugates may provide a steric barrier on the vesicle surface which prevents the close contact between the vesicles, thus minimizing the fusion-induced membrane damage. Mono- and di-saccharide conjugates would have less ability to do so due to their smaller saccharide size. Finally, the presence of the saccharide-DOPE conjugates in the membrane could also diminish the unstable domain boundaries which arise as a result of the phase transition induced by the dehydration effect of the freeze-thaw cycles. Data in Table 3-I support this hypothesis, because NANL-DOPE was more effective than Gal-DOPE in increasing the membrane disorder of DPPC bilayer at a temperature below the chain-melting temperature of DPPC. Thus, a bilayer containing NANL-DOPE is expected to be more disordered, and thus contains fewer domain boundaries than the one containing Gal-DOPE, during the phase transition induced by the freeze-thaw procedure. While we do not have sufficient experimental data to favor or disfavor any particular mechanism listed above, it is important to note that these different mechanisms are not mutually exclusive. In fact, it is likely that more than one mechanism is involved in the cryoprotectant activity of a given glycophospholipid conjugate.

Somewhat unexpected are the observations that Gal- and Lac-DOPE are antagonistic to the cryoprotective effect of a well established

cryoprotectant, i.e. trehalose (Figures 3-1,3-3). Such antagonistic effect was particularly prominent if the vesicles contained a high concentration of either Gal-DOPE or Lac-DOPE (Figure 3-1a,b), indicating that some direct interactions (or the absence of them) between the trehalose and the glycophospholipid are responsible for the observed activity. The cryoprotective activity of the trisaccharide-DOPE conjugates was synergistic to that of the trehalose (Figure 3-1). Thus, we decided to study in some detail the direct interactions of trehalose with one glycophospholipid from each group, i.e. NANL-DOPE and Gal-DOPE.

FTIR studies of the P-O asymmetric stretching band of the glycophospholipids clearly showed that the phosphodiester group of NANL-DOPE, but not Gal-DOPE, can form H-bonding with trehalose (Figure 3-4). This is a somewhat surprising result in that the trisaccharide moiety of NANL-DOPE does not seem to be a steric barrier for trehalose to reach the phosphodiester group, yet the monosaccharide group of Gal-DOPE appears to be a barrier. A reasonable guess for the mechanism of this unusual observation is that the trisaccharide-DOPE conjugate has a more flexible headgroup conformation than the monosaccharide-DOPE conjugate. Penetration of a free disaccharide such as trehalose to the phosphodiester group would be easier if the trisaccharide chain is more extended and more conformationally flexible. In line with the hypothesis is the data shown in Table 3-I. NANL-DOPE had produced a greater effect than the Gal-DOPE to disorder the rigid DPPC bilayer. This is expected if one assumes that the headgroup of NANL-DOPE is more flexible and thus occupies a greater volume than the headgroup of Gal-DOPE. Also shown in Table 1 is the ability of trehalose to partially restore the membrane order of DPPC bilayer containing NANL-

DOPE, but not the bilayer containing Gal-DOPE. It implies that the interaction of trehalose with NANL-DOPE restricts the conformational flexibility of the trisaccharide headgroup and results in a higher level of membrane order. Lower degree of trehalose interaction with Gal-DOPE is probably the reason why trehalose had no significant effect on the membrane order of the DPPC bilayer containing this glycophospholipid.

Trehalose is known to destabilize the L_α phase of the DEPE by decreasing the value of T_H (28). The proposed mechanism for this behavior of trehalose is that the disaccharide can form H-bonding with the phosphorylethanolamine headgroup and decrease the interfacial hydration of the DEPE (28). Lack of direct interaction between trehalose and Gal-DOPE would explain the additive effect of the two compounds to depress the value of T_H (Figure 3-5). On the other hand, trehalose had completely abolished the L_α phase stabilization effect of NANL-DOPE (Figure 3-5). Interaction of trehalose with NANL-DOPE must decrease the hydration level of this trisaccharide-DOPE conjugate, possibly by blocking the H-bonding with the interfacial H_2O molecules.

In summary, we have studied the cryoprotective activity of a group of synthetic glycophospholipids and found that the activity correlates well with the size, but not with the charge content, of the saccharide headgroup. Mono- and disaccharide glycophospholipids showed a concentration-dependent antagonistic effect on the cryoprotective activity of trehalose; whereas the cryoprotective activity of the trisaccharide glycophospholipids is synergistic with that of the trehalose. These observations can be understood on the basis of H-bonding between trehalose and the headgroups of the glycophospholipids. Trehalose readily interacts with trisaccharide glycophospholipid but not with the

glycophospholipids of smaller saccharide size. These conclusions should be useful in our understandings of the cryoprotective mechanism of the membrane bound carbohydrates and the future design of the effective cryoprotectants for liposomes and cells.

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Appendix

Table 3-I

Effect of glycophospholipids and trehalose on the fluorescence anisotropy of DPH in DPPC liposomes[1]

Lipid Composition	Fluorescence Anisotropy	
	PBS	trehalose[2]
DPPC	0.266 (0.002)[3,4]	0.264 (0.004)
DPPC: NANL-DOPE (3:2)	0.124 (0.003)	0.157 (0.008)
DPPC:Gal-DOPE (3:2)	0.136 (0.006)	0.133 (0.006)

[1]Anisotropy measured at the sample temperature of 33_C.

[2]Trehalose concentration was 0.1M in PBS.

[3]Mean (standard deviation), n=6 or 9.

[4]All values are significantly different from each other ($P<0.001$), except the ones for DPPC in PBS and in trehalose, and the ones for DPPC:Gal-DOPE (3:2) in PBS and in trehalose.

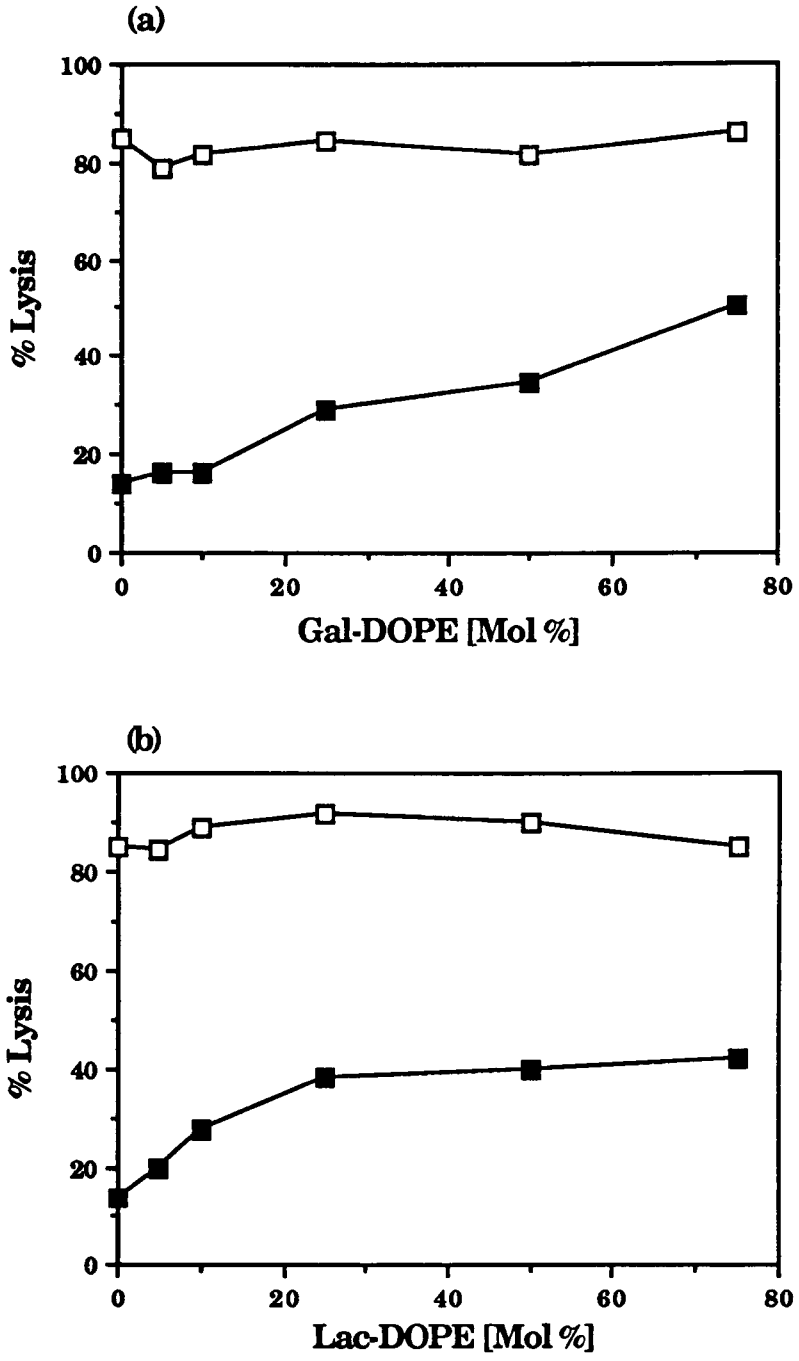
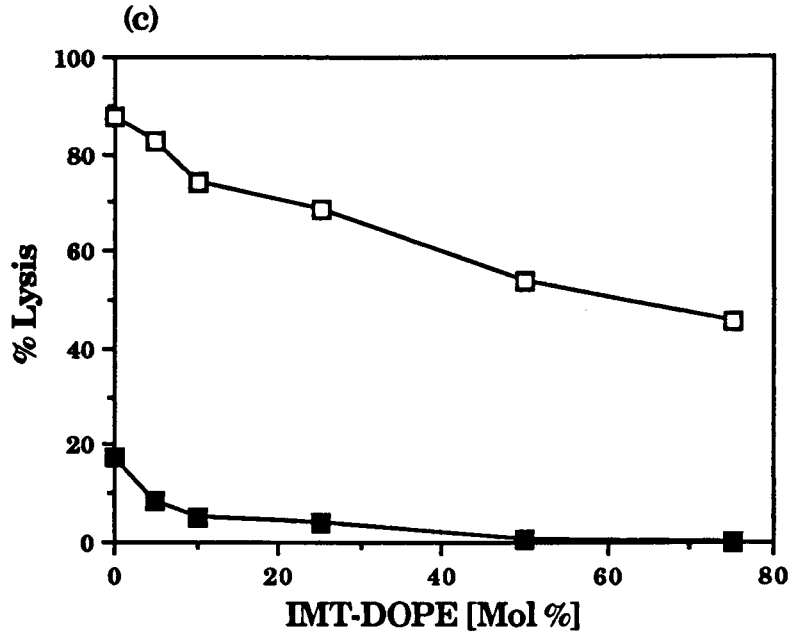


Figure 3-1. Effect of glycolipid conjugates on egg PC liposome stability during freeze-thaw cycles. Liposomes were frozen and thawed 5 times in the presence (■), or absence (❑) of 0.1 M trehalose. (a) Gal-DOPE, (b) Lac-DOPE, (c) IMT-DOPE, and (d) NANL-DOPE.



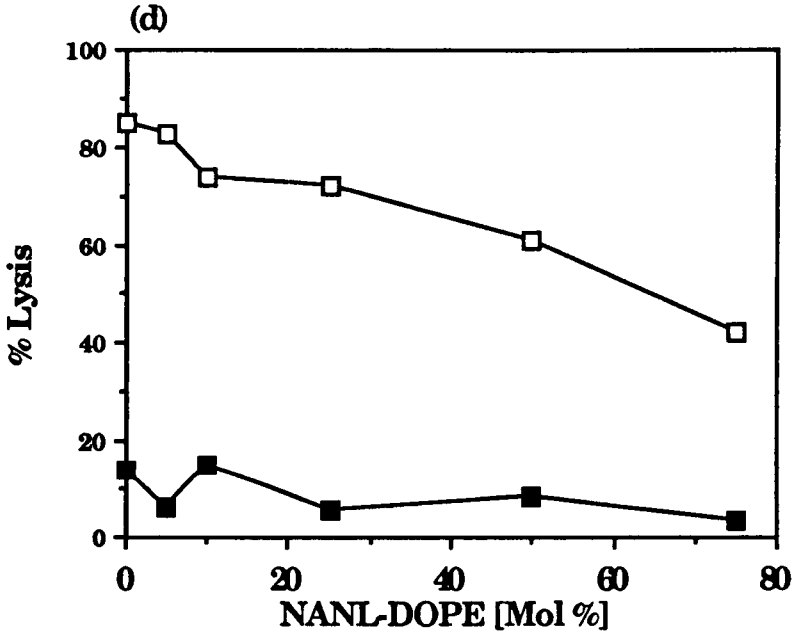


Figure 3-1. (continued)

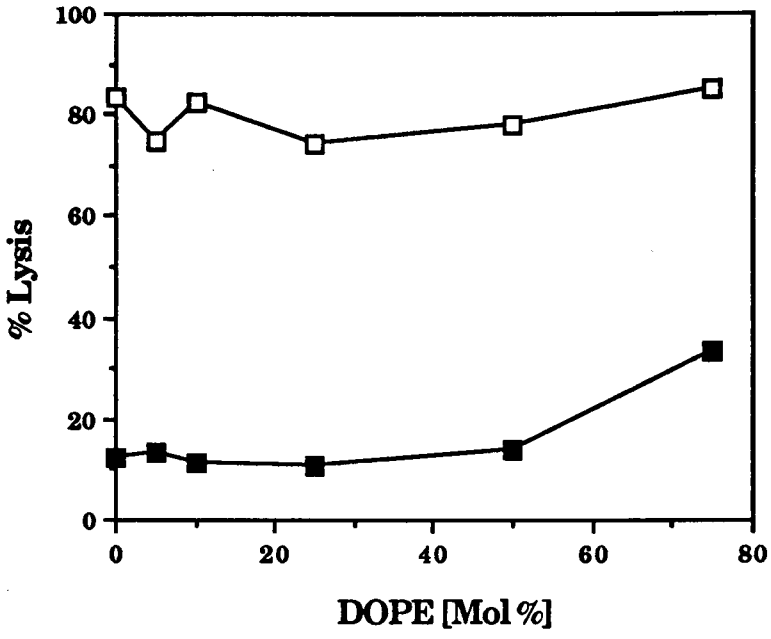


Figure 3-2. Effect of DOPE on egg PC liposome stability during freeze-thaw cycles. Liposomes were frozen and thawed 5 times in the presence (■) or absence (❑) of 0.1 M trehalose.

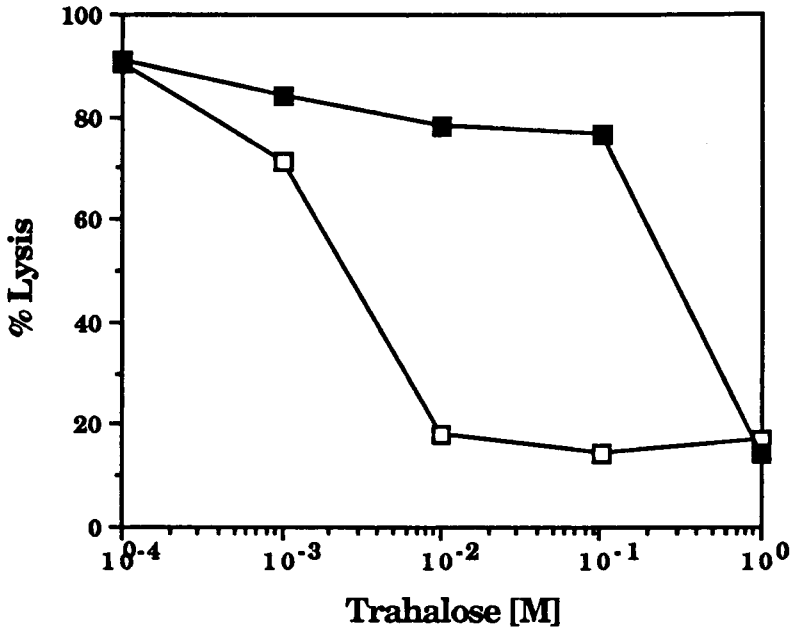


Figure 3-3. Effect of trehalose to protect liposomes composed of egg PC (■) or Gal-DOPE (❑) against the freeze-thaw damage.

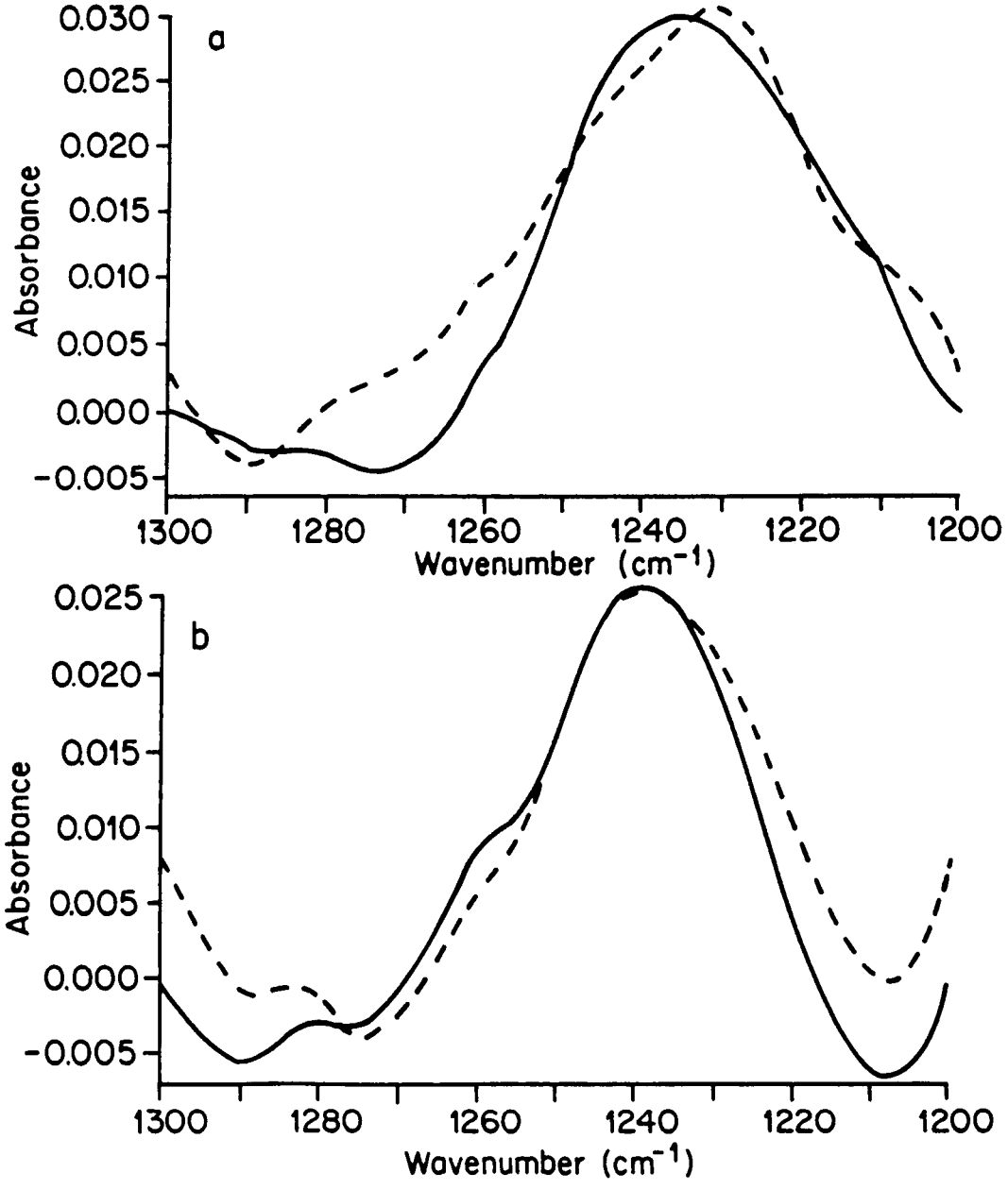


Figure 3-4. FTIR spectra of glycophospholipids. The P-O asymmetric stretching bands between 1200-1300 wave number (cm^{-1}) are shown. NANL-DOPE (a) and Gal-DOPE (b) were dried with (....) or without (__) trehalose (ratio of lipid to trehalose, 1:5).

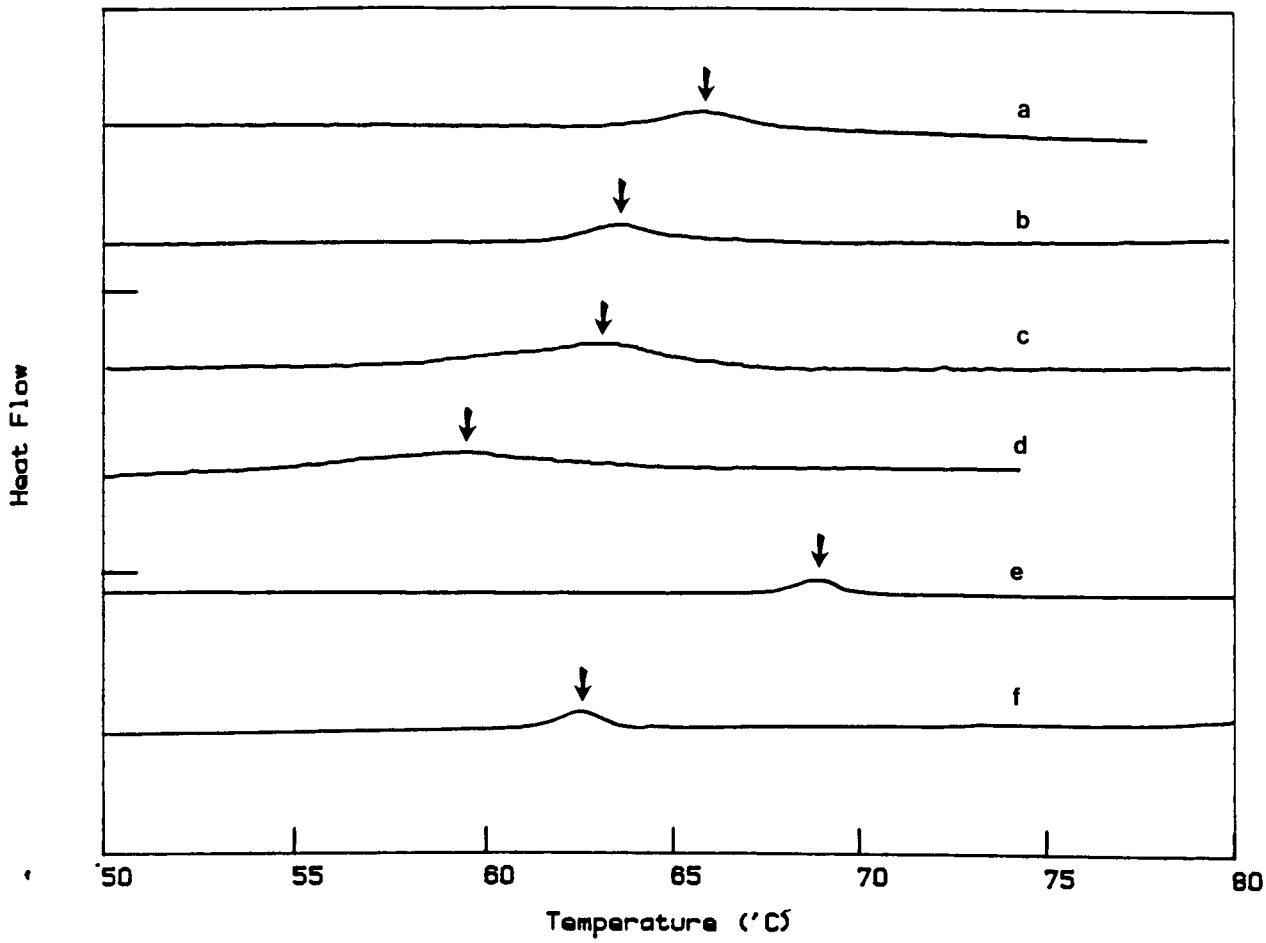


Figure 3-5. Differential scanning calorimetry of DEPE in the presence of glycophospholipids and trehalose. Excess heat capacity, cp was recorded as a function of temperatures. (a) DEPE in PBS, (b) DEPE in 0.1 M trehalose, (c) Gal-DOPE (4 mol %)/DEPE in PBS, (d) Gal-DOPE (4 mol %)/DEPE in 0.1 M trehalose (e) NANL-DOPE (3 mol %)/DEPE in PBS, (f) NANL-DOPE (3 mol %)/DEPE in 0.1 M trehalose.

PART IV

SOME NEGATIVELY CHARGED PHOSPHOLIPID DERIVATIVES PROLONG THE LIPOSOME CIRCULATION IN VIVO

1. Abstract

A series of negatively charged phospholipid derivatives has been synthesized by coupling aliphatic dicarboxylic acids, $HOOC(CH_2)_nCOOH$, to dioleoylphosphatidylethanoamine (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 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-pimeryl DOPE and N-suberyl DOPE. Our observation suggested that not all negatively charge phospholipids enhance liposome uptake by RES, some even reduce the uptake.

2. Introduction

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 differnt ways in 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), phophatidyl-glycerol (PG), and other negatively charged phospholipids are known to be typical lipid components which 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. "shielded negative charge" hypothesis (2,3).

In this report 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.

3. Materials and methods

a. Materials

Egg PC and DOPE were purchased from Avanti Polar Lipid (Birmingham, AL). Malonic acid, succinyl anhydride, glutaryl anhydride, adipic acid, pimelic acid, suberic acid, and cholesterol were purchased from Sigma Chemical Co. The synthesis of DTPA-SA has been described (13). 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.

b. Synthesis of DOPE derivatives

Some of the DOPE derivatives were prepared by coupling dicarboxyl ligands to DOPE via dicyclohexylcarbodiimide (DCC) activation (14). 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 approximately 60 % after 4 h incubation. The rest of DOPE derivatives were prepared from anhydrides of dicarboxylic acid and DOPE as previously described (15). 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). The final reaction mixture was dried in a rotatory evaporator and then disssolved 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 (16). Partial characterization of DOPE derivatives was done by HPTLC and infrared spectroscopy. The purified DOPE derivatives appeared to be a single spot on HPTLC plate with the solvent system mentioned above. The 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 (data not shown). The appearance of O-H stretching of carboxylic acid was indicated by infrared absorbance at 3000 cm^{-1} (data not shown).

c. Liposome preparation

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 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 analyser (Hialeah, FL) was approximately 175 nm.

d. Biodistribution studies

^{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 sacrificed by cervical dislocation at 3 h post injection. Three 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 approximately 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).

e. Liposome agglutination by streptavidin

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). Fifty μl liposomes (1 mg lipid/ml) were mixed with 0.5 ml PBS in a microcuvette, and 5 μg streptoavidin was added. Increase in turbidity was monitored as the optical density at 440 nm.

4. Results and Discussion

The purified negatively charged DOPE derivatives were incorporated into egg PC/cholesterol liposomes which were then i.v. injected into mice to examine the effect of DOPE derivatives on liposome circulation. 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 4-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 (Figure 4-1). 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 liposomes cleatance 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 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 proton NMR studies indicates that the G_{M1} structure is stabilized by an interaction between sialic acid and the galactosamine residue (19,20). Other evidence on head group 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(ethyleneglycol)-phospholipid conjugates (5) which contain a negatively charged phosphate group shielded by a poly(ethyleneglycol) 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 the hydroxyl groups of G_{M1} and the poly(ethyleneglycol) 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. We have previously shown that such measurement sensitively reveals the steric barrier activity of G_{M1} and the poly(ethyleneglycol) 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} (Figure 4-2).

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 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 dysposonin 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 head group due to the flexibility of the hydrocarbon chain. These carboxyl groups would not be available for the dysopsonin recognition.

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Appendix

Table 4-I

Biodistribution of liposomes containing negatively charged DOPE derivatives[1]

Lipids	^{111}In Distribution (% injected dose)[2]		
	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)

[1]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 sacrificed 3 h later.

[2]Data represent mean (S.D.),n=3.

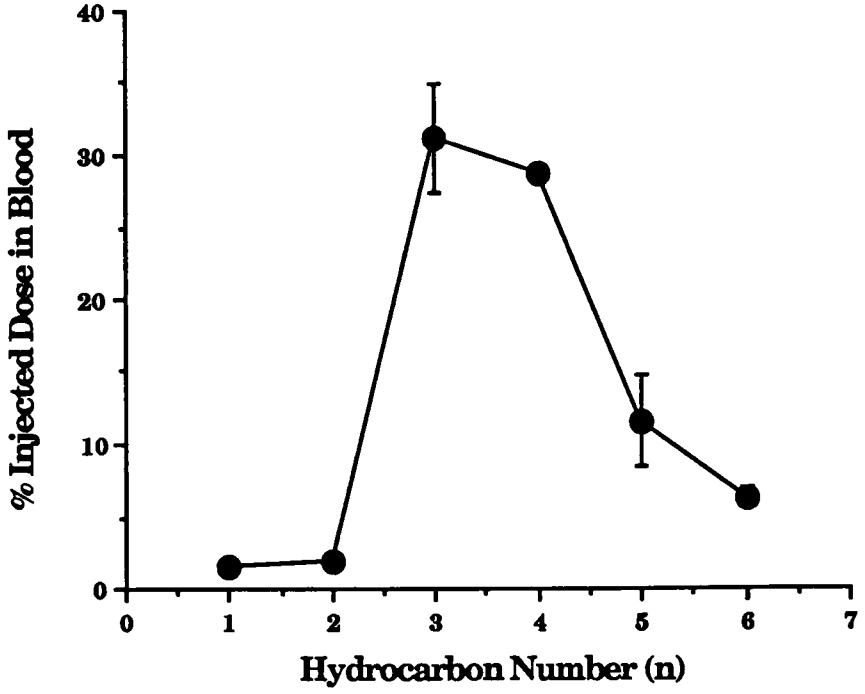

Figure 4-1. Effect of DOPE derivatives on liposome concetration in the blood Liposomes composed of egg PC:cholesterol: DOPE derivatives (10:5:1) were labeled with [^{111}In]DTPA-SA and injected into mice. Percent injected dose in blood was measured 3 h post injection and plotted against the hydrocarbon number (n) of the general formula, DOPE-NH-CO-$(CH_2)_n$-COOH.

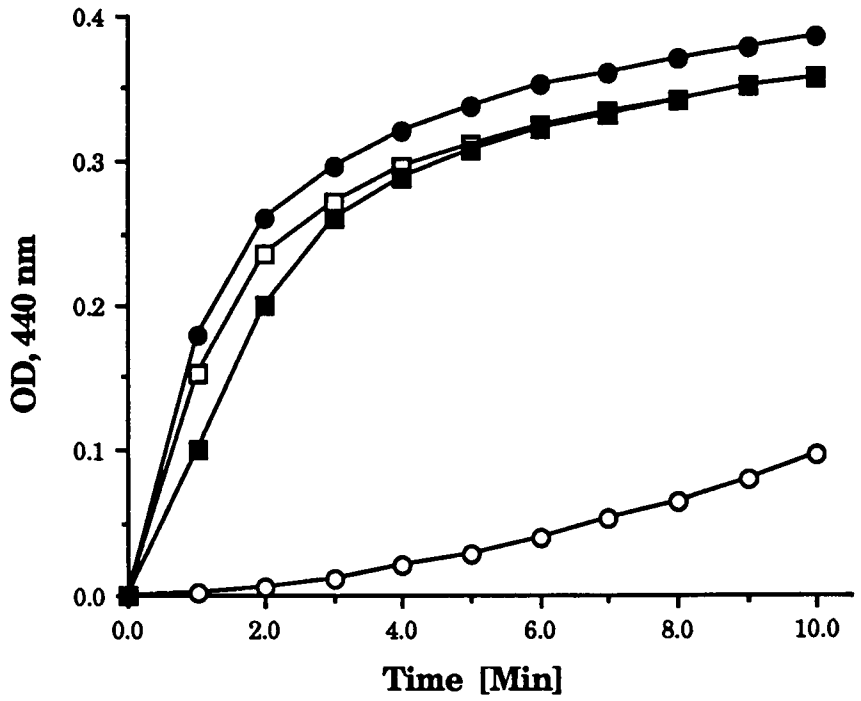

Figure 4-2. Effect of NGPE and NAPE on the streptavidin-induced agglutination of liposome containing N-biotinyl-PE. The turbidity increase (A_{440}) was measured with time for liposomes containing none (●), G_{M1} (○),NGPE (■), and NAPE (❑).

PART V

EFFECT OF CHEMICALLY MODIFIED GM1 AND NEOGLYCOLIPID ANALOGS OF GM1 ON LIPOSOME CIRCULATION TIME: EVIDENCE SUPPORTING THE DYSOPSONIN HYPOTHESIS

1. Abstract

The sugar group of ganglioside G_{M1} has been modified by periodate oxidation, reduction, or reductive amination. The negative charge of sialic acid of G_{M1} has also been removed by methylation or reductive hydrolysis. A series of neoglycolipid analogs of G_{M1} was synthesized by coupling the G_{M1} oligosaccharide (G_{M1}OS) to dioleoylphosphatidylethanolamine (DOPE) via different spacer arms. The individual G_{M1} derivative was incorporated into egg phosphatidylcholine/cholesterol liposomes and tested in mice in order to see whether it was effective in prolonging liposome circulation. The oxidized G_{M1} did not show any activity in prolonging circulation. However, the lost activity after oxidation was completely recovered by the subsequent reduction step. After oxidation followed by reductive amination with various substituted amines, a series of aminated G_{M1} derivatives were prepared. β-Alanyl G_{M1} showed a quite comparable activity to the native G_{M1} while other aminated G_{M1}'s showed reduced activity in terms of prolonging circulation of liposomes. Blocking the negative charge of sialic acid by methylation did not deprive the activity and removing the carboxyl group of sialic acid by reductive hydrolysis reduced the activity by only approximately 20 %. Among neoglycolipid analogs of G_{M1}, only G_{M1}OS directly conjugated to DOPE was effective in prolonging the circulation, whereas conjugates with a spacer of various length were not effective. These results emphasize the importance of molecular structure of G_{M1} for its functional activity in prolonging the liposome circulation. Furthermore, modifications which abolish the cholera toxin binding activity of G_{M1} also decrease the activity to prolong the circulation time of liposomes, and vice versa. Such strong correlation further supports the

idea that specific recognition of G_{M1} oligosaccharide by putative " dysopsonin(s)" is responsible for the activity to prolong the liposomes circulation time.

2. Introduction

Liposomes as an in vivo drug delivery system have been developed and improved to enhance drug therapy (for review, see refs. 1 and 2). Generally the clinical applications of liposome are based on two different rationales : controlled drug-release (3) and site-directed delivery (4). Most of systemically administrated liposomes are rapidly taken up by reticuloendothelial system (RES) cells in liver and spleen, and the RES cells may release the drugs entrapped in liposomes slowly and continuously (1,5). The controlled drug-release by RES enhances therapeutic efficacy and reduces toxicity of various drugs (3). However, the avid RES uptake of liposomes substantially reduces their target binding. For example, accumulation of liposomes in the solid tumor is generally very low, but can be significantly enhanced if the RES is temporarily blocked by preinjection of empty liposomes or other RES blockade agents (6).

Allen and coworkers have found that ganglioside G_{M1} reduces the rate and extent of liposome uptake by the RES and thereby prolongs circulation time of liposomes (7,8). Gabizon and Papahadjopoulos have further examined a number of other natural and synthetic lipids, and found that phosphatidylinositol and sulfatides also reduce the RES uptake of liposomes (9). They have proposed a mechanism concerning the effect of G_{M1} on the interactions between liposomes and the RES cells. The negative charge of G_{M1} which is shielded by a bulky, neutral, and hydrophilic

oligosaccaride group may contribute to the RES avoidance by decreasing or preventing opsonization of the liposomes, or direct binding to the cell-surface receptors of the macrophage.

We have performed a structure-function study on G_{M1} by chemically modifying the oligosaccharide structure of G_{M1}. We have also synthesized a series of neoglycolipid analog of G_{M1}. These compounds were incorporated into liposomes and tested their activities to prolong the circulation time of liposomes. These studies have yielded usefull information about the structure feature of G_{M1} which is important for the RES avoidance activity and should shed light on the mechanism of action of this important ganglioside.

3. Materials and methods

a. Materials

Egg PC was purchased from Avanti Polar Lipids (Birmingham, AL). Ganglioside G_{M1} was purchased from Matreya, Inc (Pleasant Gap, PA). β-Alanine, ethanolamine, N-methylethylenediamine, N,N-dimethylethylene - diamine, ethylenediamine, iodomethane, and cholesterol were purchased from Sigma Chemical Co. The synthesis of DTPA-SA has been described (10). The [^{111}In]DTPA-SA was prepared by mixing 1 μl of $^{111}InCl_3 \cdot HCl$ with 600 ul of 1mM DTPA-SA in ethanol at 80 °C with brief sonication.

b. Chemical modification of G_{M1}

Chemical modifications of G_{M1} are shown in Figure 5-1a. The preparation of oxidized $C7G_{M1}$ has been described (11,12). G_{M1} in 50 mM

sodium acetate containing 0.14 M NaCl (pH 4.5) was mixed with $NaIO_4$. The molar ratio of G_{M1} to periodate was 1:10 and the final concentration of $NaIO_4$ was below 0.1 M. The reaction mixture was incubated in the dark and at 0~2 °C for 12 h. An excess amount of sodium metasulfite was added to stop the oxidation. One tenth of the reaction mixture was extensively dialyzed against water and lyophilized. The rest was dialyzed against 50 mM phosphate containing 0.14 M NaCl (pH 7.5) for further modification. The oxidized $C7G_{M1}$ was converted to the reduced $C7G_{M1}$ after incubation with $NaBH_4$ (100:1, molar ratio of $NaBH_4$ to $C7G_{M1}$) for 1 h at room temperature and the reduced $C7G_{M1}$ was extensively dialyzed against water. β-Alanine, ethanolamine, N-methylethylenediamine, N,N-dimethylethylene-diamine, or ethylamine was mixed with the oxidized $C7G_{M1}$ (molar ratio of the amine to $C7G_{M1}$, 10:1) and incubated in the presence of excess $NaCNBH_3$ (100:1, molar ratio of $NaCNBH_3$ to ligand) for 3 h at room temperature (13). The aminated $C7G_{M1}$'s were extensively dialyzed against water and lyophilized. The G_{M1} derivatives were run on a high performance thin-layer silca gel plate (Whatman) with a solvent system of $CHCl_3$:MeOH:0.2% $CaCl_2$ (50:45:5) and developed by resorcinol reagent (Figure 5-2a). Also, the derivatives were spotted on Whatman No. 1 paper and placed in a sealed electrophoresis chamber. The samples were electrophoresed in methanol/50 mM Tris (pH 8.5)(10:1, v/v) for 30 min at 500 V and room temperature and then developed by iodine vapor (Figure 5-2b).

c. Methylation and reduction of G_{M1}

Methylation and reduction of G_{M1} are shown in Figure 5-1a. The carboxyl group of sialic acid was esterified according to the method

described by Handa and Nakamura (14). G_{M1} was incubated at room temperature for 30 min in a mixture of dimethylsulfoxide and iodomethane (5:1, v/v). After evaporation, the G_{M1} mixture was dissolved in methanol and then applied to a DEAE-Sephadex column, equilibrated with methanol, to remove unmethylated G_{M1}. The collected fractions containing methylated G_{M1} were extensively dialyzed against water and lyophilized. The methyl ester of sialic acid was further reduced by incubating at room temperature for 4 h in methanol containing 0.5 % $NaBH_4$. The methylated and the reduced G_{M1} were characterized by thin-layer chromatography (Figure 5-2c) and paper electrophoresis (Figure 5-2d) described earlier, and by infrared spectroscopy (14).

d. Synthesis of neoglycolipid analogs of G_{M1}

Structures of neoglycolipid analogs of G_{M1} are shown in Figure 5-1b. Preparation of the oligosaccharide moiety of G_{M1} ($G_{M1}OS$) has been described by Pacuszka and Fishman (15). Briefly, A stream of ozone in oxygen from an ozone generator was passed through a G_{M1} solution in methanol (0.1 g ozone/50 mg G_{M1}). After evaporation the residue was dissolved in deaerated 1 M Na_2CO_3 (pH 9.0) and incubated at room temperature for 18 h. $G_{M1}OS$ was purified by anion exchange chromatography (Dowex AG1-X2, acetate form). The procedure of synthesis of $G_{M1}OS$-DOPE was described by Stoll et al (16). $G_{M1}OS$ was coupled to DOPE via reductive amination in the presence of an excess $NaCNBH_3$.

The synthesis procedure of $G_{M1}OSNH$-X-DOPE and $G_{M1}OSNHC8NH$-X-DOPE (Figure 5-1b) was described by Pacuszka et al. (17). First, $G_{M1}OS$

was reductively aminated to $G_{M1}OSNH_2$ (18). $G_{M1}OS$ was dissolved in 20 % aqueous NH_3 (2 mg/ml) and incubated for 16 h at 50 °C. After it was cooled to -40 °C, the reaction mixture was incubated for 6 h at room temperature in the presence of excess $NaCNBH_3$. The reaction mixture was acidified by acetic acid to quench the remaining $NaCNBH_3$. For the preparation of $G_{M1}OSNHC8NH_2$, $G_{M1}OS$ was dissolved in methanol containing 1,8-diaminooctane (molar ratio of $G_{M1}OS$ to diaminooctane, 1:50). After 2 h incubation at 50 °C, excess $NaCNBH_3$ was added and the reaction was allowed to proceed for 40 h at 50 °C. An excess amount of acetic acid was added to remove the remaining $NaCNBH_3$. Both preparations were applied to a Sephadex G15 column to remove salts and ions. $G_{M1}OSNH_2$ (or $G_{M1}OSNHC8NH_2$) and DOPE (molar ratio, 1:10) were dissolved in anhydrous chloroform/methanol (1:1, v/v) containing 0.1 % triethylamine, and sonicated for 5 min. Disuccinimidyl suberate (DSS) was slowly added stepwise with stirring for 3 h until a molar ratio of $G_{M1}OSNH_2$ (or $G_{M1}OSNHC8NH_2$) to DSS of 1:5 was reached. The reaction mixture was incubated overnight with stirring. Each reaction preparation ($G_{M1}OS$-DOPE, $G_{M1}OSNH$-X-DOPE, and $G_{M1}OSNHC8NH$-X-DOPE) was applied to a DEAE-Sephadex column equilibrated with methanol. The neoglycolipids were eluted by an acetate gradient (0 to 0.15 M). The purified neoglycolpids were extensively dialyzed against water and lyophilized. The neoglycolipids of G_{M1} were characterized by thin-layer chromatography described earlier (Figure 5-2e) and by infrared spectroscopy, and quantitated by resorcinol assay (19) and phosphate assay (20).

d. Liposome preparation

Liposomes were prepared from egg PC, cholesterol, and G_{M1} derivatives (molar ratio, 10:5:1) with a radioactive tracer marker ^{111}In-DTPA-SA. Each lipid mixture was dried under a stream of N_2 gas followed by vacuum dessication for over 1 h. PBS (pH 7.4) was added and the lipids were hydrated for 1 h at room temperature. The lipid suspension was rigorously vortexed and extruded through two stacked Nuclepore polycarbonate filter (five times through 0.4 μm filters and ten times through 0.2 μm filters). Liposome size was estimated with a Coulter N4SD sub-micron particle analyzer (Hialeah, Fl).

e. Biodistribution studies

Biodistribution studies were performed on female ICR mice weighing 22~27 g. Liposomes (0.2 mg lipid) labeled with ^{111}In were injected via the tail vein. At 3 h post injection the mice were anesthetized with Metofane (Pitman-Moore, NJ) and sacrificed by cervical dislocation. Three hour time point was chosen because $t_{1/2}$ of liposome clearance are generally between <1 and 7 h for liposomes composed mainly of egg PC and cholesterol and having an average diameter of 200 nm (21). Blood and other internal organs were collected separately, weighed, and ^{111}In radioactivity was counted in a gamma counter. Weight of mouse blood was assumed to be 7.3 % of the body weight. The dose in the internal organs was corrected for the blood contamination (22). The degree of correction was small, being 0.01 % and 7.8 % for spleen and liver, respectively.

4. Results

a. Partial characterization of G_{M1} derivatives

Chemical modification of G_{M1}'s was monitored by HPTLC (Figure 5-2a). The oxidized $C7G_{M1}$ had higher mobility than G_{M1} in the solvent system, $CHCl_3$:MeOH:0.2 % $CaCl_2$ (50:45:5) because it had fewer hydroxyl groups than the native G_{M1}. The reduced $C7G_{M1}$ migrated between G_{M1} and the oxidized $C7G_{M1}$ in the same solvent system. It is well documented that the periodate oxidation condition chosen for this preparation should attack the polyhydroxyl side chain of sialic acid although periodate ion is also capable of cleaving other C-C bonds which contain vicinal hydroxyl groups. This might be case because, after reductive amination of the oxidized G_{M1}, some minor bands were seen on the HPTLC plate. Paper electrophoresis was also used to characterize the modifed G_{M1} (Figure 5-1b). The oxidized $C7G_{M1}$ and aminoethyl G_{M1} showed slow migration to the anode, compared with the native G_{M1}, the reduced $C7G_{M1}$, or hydroxyethylamino G_{M1} (Figure 5-2b). This may be due to the increased hydrophobicity of the oxidized G_{M1} because of the loss of the polyhydroxyl side chain, which results in a lower solubility in the solvent system. The reduced G_{M1} migrated approximately the same distance as G_{M1}. β-Alanyl G_{M1} showed a little greater and more diffused migration due to the addition of negative charge. After reductive amination with diaminocompounds, the migration patterns of G_{M1} derivatives had clearly changed. These G_{M1} derivatives were mainly located at the origin since they were neutralized by addition of positive charges. In the case of G_{M1} coupled to diamino ligands, some minor spots were seen at the cathode area. According to this result, the oxidized $C7G_{M1}$ appeared to contain minor impurities which probably contain more than

one aldehyde group after oxidation.

The methylated G_{M1} and the reduced $C9G_{M1}$ purified from DEAE-Sephadex column were located on HPTLC plate as a clear single band at a farther position than G_{M1} (Figure 5-2c). It was due to the loss of the negative charge by methylation, and the loss of carboxyl group via reductive hydrolysis resulted in an increase of hydrophobicity of the methylated G_{M1} and the reduced $C9G_{M1}$, respectively. The formation and disappearance of the carboxy ester bond by methylation and reduction were also indicated by appearance and disappearance of an absorption at 1745 cm^{-1} on infrared spectra, respectively (data not shown). The neoglycolipid analogs of G_{M1} were also visualized as single bands on a HPTLC plate (Figure 5-2d). G_{M1}OS-DOPE migrated slower than the native G_{M1} since DOPE were less hydrophobic than the ceremide moiety of G_{M1}. The insertion of a succinyl spacer arm to G_{M1}OS-DOPE made the analog, G_{M1}OS-NH-X-DOPE, more hydrophilic, resulting in a much slower migration in HPTLC. However, G_{M1}OSNHC8NH-X-DOPE was as hydrophobic as the native G_{M1} because of the addition of an octanyl group. Phosphate assay (19) and resorcinol assay (20) were performed to quantitate phosphate and sialic acid of the neoglycolipids, respectively. For each analog the molar ratio of sialic acid to phosphate was close to 1. Additionally, the appearance of 1150 cm^{-1} absorption on infrared spectra indicated the formation of an amide bond (data not shown).

b. Effects of chemically modified G_{M1} on liposome biodistribution in vivo

Even though some of the G_{M1} derivatives contained minor impurities, they were incorporated into egg PC/cholesterol liposomes without further

purification. Homogeneous ^{111}In-labeled liposomes with an average diameter of 175 +35 nm were prepared by the extrusion method (23). The liposomes containing various G_{M1} derivatives were i.v. injected into mice via tail vein and the biodistribution was measured 3 h later. Table 5-I shows % of the injected dose in blood, spleen, and liver where were the three major compartments containing the injected liposomes. The oxidation of sialic acid abolished the activity of G_{M1} in prolonging the liposome circulation. The amount of liposomes containing the oxidized $C7G_{M1}$ in the blood at 3 h postinjection was only 5 % of the injected dose comparing to 58 % for liposomes containing the native G_{M1}. The reduction of liposome concentration in the blood was accompanied by a concomitant increase in the liver and spleen uptake. However, subsequent reduction step had significantly increased the activity of the oxidized $C7G_{M1}$ to a level similar to that of the native G_{M1}. Among the reductively aminated G_{M1} derivatives, β-alaninyl G_{M1} also showed a quite comparable activity to G_{M1}. hydroxyethylamino G_{M1} and aminoethyl G_{M1} showed over 80 % activity as compared with the native G_{M1}. Coupling diaminocompounds such as ethylenediamine, N-methylethylenediamine, or N,N-dimethylethylenediamine, to the oxidized G_{M1} failed to fully restore the lost activity after oxidation. These results clearly indicate that the substitution of the polyhydroxyl side chain of sialic acid is critical for the G_{M1} function in prolonging the liposome circulation.

In order to examine the function of the carboxyl group of sialic acid, we synthesized the electrically neutral G_{M1} derivatives by methylation or reductive hydrolysis. These G_{M1} derivatives were also tested for the activity to prolong the liposome circulation (Table 5-II). Methylation on the carboxyl group of G_{M1} did not affect the G_{M1} function in terms of prolonging

circulation of liposomes. Removal of the carboxyl group of sialic acid reduced the activity by only less than 20 %. These results indicate that the carboxyl group of sialic acid is not critical for the G_{M1} function in prolonging the liposome circulation.

c. Effects of neoglycolipid analogs of G_{M1} on liposome biodistribution

Recently, Pacuszka and coworkers have reported that the distance between the olgosaccharide of G_{M1} and the phospholipid appears to influence its functional activity in terms of binding to cholera toxin (17). In order to examine whether the distance of oligosaccharide moiety from the phospholipid bilayer surface affects the G_{M1} function in prolonging the liposome circulation, we synthesized a series of neoglycolipid analogs of G_{M1} such as G_{M1}OS-DOPE, G_{M1}OSNH-X-DOPE, and G_{M1}OSNHC8NH-X-DOPE (Figure 5-1b). Table 5-III shows the biodistribution of liposomes containing various neoglycolipids 3 h post injection. The neoglycolipid synthesized by directly attaching G_{M1}OS to DOPE was effective at approximately 70 % activity of the native G_{M1} in prolonging circulation of liposomes, whereas the others synthesized by cross-linking with DSS and/or diaminooctane were not. These results indicate that the oligosaccharide of G_{M1} needs to be physically close to the bilayer surface for the activity to prolong the circulation time of liposomes; neoglycolipids with longer spacer arms were not effective.

5. Discusion

There are generally two classes of amphiphiles which prolong liposome circulation time by reducing the RES uptake. Hydrophilic, long-

chain polymers such as poly(ethyleneglycol) exhibit the activity by presenting a steric barrier on the liposome surface and protecting the liposomes from interaction with opsonin(s) and/or other inactivating factors (24). The other class of amphiphile consists of naturally occurring glycolipids such as G_{M1}, phosphatidylinositol, and sulfatides (9). We have previously shown that the activity of G_{M1} cannot be fully accounted for by the weak steric barrier activity of this glycolipid (25); an additional or alternative mechanism has to be considered.

According to carbon-13 and proton NMR studies, G_{M1} structure appears to be stabilized by an interaction between sialic acid and the galactosamine residue in the oligosaccharide portion (26,27). Studies on head group conformation of G_{M1} suggest that the carboxyl group of sialic acid forms an internal chelation complex with some hydroxyl group of the galactose residue (28). From this point of view, a mechanism for the function of G_{M1} in the interaction between liposome and RES has been proposed, that shielding the negative charge of G_{M1} by a bulky hydrophilic, neutral sugar moiety may contribute to the RES avoidance activity by decreasing or preventing opsonization of liposomes (8,9). Although this "shielded negative charge" hypothesis is consistent with the activity of poly(ethyleneglycol)-phosphatidylethanolamine conjugates to prolong the liposome circulation time (29,30), data presented in this report are not consistent with the hypothesis. First, G_{M1} derivatives with the carboxyl group methylated or hydrolyzed by reduction still show considerable activity in prolonging the circulation time of liposomes (Table 5-II). In other words, the negatively charged carboxyl group is not essential for the activity. Reductive amination of the oxidized $C7G_{M1}$ in the presence of β-alanine adds to the G_{M1} oligosaccharide an additional carboxyl group which

is not necessarily "shielded" by the neutral sugar residues, yet the β-alaninyl G_{M1} had an activity very similar to that of the native G_{M1} (Table 5-I). Furthermore, we have recently shown that some aliphatic dicarboxylic acids conjugates to DOPE exhibited considerable activities to prolong the liposome circulation time (31). Also, it has been recently reported that glucronic acid coupled to palmitate prolongs the circulation time of liposomes compared with the liposomes containing DPPG instead of palmityl glucronic acid (32). The carboxyl group of these conjugates is located at the end of the head group and is obviously not "shielded". These evidence taken together argue strongly against the "shielded negative charge" hypothesis for the activity of G_{M1}.

From the viewpoint of structure-activity relationship, our data indicate that the polyhydroxyl side chain of the sialic acid is much more important than the carboxyl group. Oxidized $C7G_{M1}$ which has lost the polyhydroxyl side chain showed no activity to prolong the circulation time of liposomes. The activity was restored to a different degree when various functional groups were added back to this position by reduction or reductive amination (Table 5-I). Addition of hydroxyl and carboxyl groups showed full activities, whereas ethyl group and various amines (1°, 2°, and 3°) had only partial activities. Previous studies have also revealed some structure specificity for the activity of G_{M1} to prolong the liposome circulation time. For example, removal of the entire sialic acid residue, i.e. the asialo G_{M1}, significantly reduces the activity of G_{M1} (7,8). Additional sialic acid residues in the oligosaccharide structure, such as those of G_{D1a} (33) and G_{T1b}, (9) also reduced the activity to prolong the circulation time. Taken together, these results strongly suggest that a specific oligosaccharide structure of G_{M1} is recognized for the activity to reduce the RES uptake of

liposomes, resulting in the prolonged circulation times.

One possibility is that such recognition is by specific serum protein(s). Binding of the G_{M1}-specific protein(s) to liposomes would lead to a reduced level of opsonization and/or inactivation of bound opsonin(s), resulting in a reduced uptake by the RES. By definition, these proteins would be named "dysopsonins" (34). Although the activity of dysopsonin has been demonstrated (34), it is not known if they are in any way related to the G_{M1} recognition.

The observation made from the chemical modification on polyhydroxyl side chain of G_{M1} appears to be related to some previously reported results. The alternative pathway of complement activation is an important recognition system by which pathogenic microorganisms are opsonized with C3b before the induction of antibody response (35). It has been shown that injected liposomes are recognized by activated complement components via the classical or alternative pathway, depending on the liposome composition (36-38). The complement coated liposomes are rapidly taken up by the RES (39). Recently, a number of reports have shown that G_{M1} can inhibit the alternative pathway mediated activation of complement (40-42), whereas the the sialic acid modified G_{M1} is not effective in this acitvity (12,43). Fearon (43) has reported that after peroxidate cleavage of the polyhydroxyl side chain, sialic acid on sheep erythrocytes could not inhibit the alternative pathway of human complement while unmodified sialic acid could. Also, Michalek et al. (12) have claimed that the inhibitory activity of G_{M1} in alternative pathway activation is abolished after removal of the C8 carbon of sialic acid. Their interpetation was that the removal of C8 carbon of the polyhydroxyl side chain was functionally equivalent to the removal of the entire sialic acid with sialidase. Our

observation was not consistent with that of Michalek et al. because the reduced $C7G_{M1}$ as well as several reductively aminated G_{M1} derivatives are active in prolonging the circulation time of liposomes (see Table 5-IV for comparison). Therefore, the activity of G_{M1} may not be related to the inhibitory activity to the complement activation via the alternative pathway.

Recent reports about cholera toxin which specifically bind to G_{M1} appear to be related to our results. It has been well documented that sialic acid is required for cholera toxin to bind to G_{M1} (44,45). The reduced $C7G_{M1}$ is as effective as the native G_{M1} in terms of cholera toxin binding, and the replacement of the polyhydroxyl side chain of sialic acid with an ethanolamine residue (45) or Lucifer yellow CH (46) does not alter the toxin binding affinity. The carboxyl group of sialic acid is not an absolute requirement for toxin binding (45). The effect of chemically modified G_{M1} on the cholera toxin binding shows a very similar pattern of effects on the liposome circulation. Finally, the substitution of the ceremide moiety with phosphatidylethanolamine reduces the toxin binding by a certain amount (17). However, G_{M1}OS-PE was the most effective among the neoglycolipid analogs of G_{M1} and the insertion of a spacer between the phospholipid and oligosacchride moiety diminishes the cholera toxin binding activity. Thus, the action of the neoglycolipid analogs of G_{M1} in the cholera toxin binding shows a very strong similarity to the action in the liposome circulation (See Table 5-IV for comparison). We speculate that the putative G_{M1}-specific dysopsonin(s) may bind to G_{M1} in a manner similar to that of cholera toxin.

Recent evidence indicates that G_{M1} containing liposomes show a greatly reduced level of protein coating as compared to liposomes without G_{M1} when exposed to blood components in vitro or <u>in vivo</u> (47). Presumably at least some of these proteins function as opsonins to promote liposome

uptake by the RES. This activity of G_{M1} can not be accounted for by the weak steric barrier activity of G_{M1} (25). Furthermore, grafting the oligosaccharide of G_{M1} to the headgroup of PE with or without an additional spacer should increase the steric barrier activity of G_{M1}, yet the activity of prolonging the circulation time of liposomes is significantly reduced or abolished (Table 5-III). On the other hand, binding of the putative dysopsonin to the G_{M1} oligosaccharide some distance away from the bilayer surface may not be efficient in preventing the coating of opsonins directly to the bilayer surface. These speculations can only be tested when the putative dysopsonin is identified.

Finally, it should be mentioned that the activity of G_{M1} in reducing liposome's affinity to macrophages may also be related to the degree of lipid peroxidation in the liposome membrane. Recently, it has been reported that a very low amount of G_{M1} in biomembranes is able to protect the membrane from lipid peroxidation (48,49). Products of lipid peroxidation are efficient chemoattractants for macrophages (50). Inhibition of lipid peroxidation by G_{M1} would reduce the production of these chemoattractants and diminish the uptake of liposomes by macrophages. This hypothesis will be tested in future experiments.

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Table 5-I

Biodistribution of lipoosomes containing chemically modified G_{M1}[1]

Chemical modification of G_{M1}			^{111}In Distribution (% injected dose)[2]		
Oxidation	Reduction	Reductive amination	Blood	Spleen	Liver
-	-	-	58.0 (1.4)	6.4 (0.6)	20.5 (0.1)
+	-	-	4.9 (0.7)	14.0 (2.5)	69.8 (3.4)
+	+	-	58.3 (2.1)	5.3 (0.8)	16.7 (0.7)
+	-	ethanolamine	52.8 (2.2)	5.7 (0.2)	25.2 (1.8)
+	-	β-alanine	60.0 (2.3)	5.7 (0.3)	22.2 (0.7)
+	-	ethylamine	46.0 (1.9)	11.1 (1.7)	29.2 (2.2)
+	-	ethylenediamine	12.3 (1.7)	18.6 (2.8)	60.8 (4.2)
+	-	N-methylethylene diamine	20.3 (3.4)	17.9 (1.8)	49.7 (4.7)
+	-	N,N-dimethyl ethylenediamine	24.9 (2.7)	17.1 (0.1)	49.0 (1.1)
no G_{M1} derivatives			3.8 (0.1)	11.1 (0.1)	68.8 (2.7)

[1]Liposomes composed of egg PC:cholesterol:G_{M1} derivatives (10:5:1) were labeled with ^{111}In-DTPA-SA. 0.2 mg liposomes were injected into each mouse which was sacrificed 3 h after injection.

[2]Data are expressed as mean (S.D.), n=3.

Table 5-II

Biodistribution of liposomes containing carboxyl-modified G_{M1}[1]

G_{M1} Derivatives	^{111}In Distribution (% injected dose)[2]		
	Blood	Spleen	Liver
G_{M1}	53.6 (5.5)	4.4 (1.7)	22.1 (3.1)
Methylated G_{M1}	49.5 (3.6)	7.5 (1.2)	26.1 (3.9)
Reduced C9G_{M1}	42.5 (2.2)	9.9 (2.1)	29.1 (4.5)
none	6.7 (1.6)	14.4 (5.3)	61.2 (3.4)

[1]Liposomes composed of egg PC:cholesterol:G_{M1} derivatives (10:5:1) were labeled with ^{111}In-DTPA-SA. 0.2 mg liposomes were injected into each mouse which was sacrificed 3 h after injection.

[2]Data are expressed as mean (S.D.), n=3.

Table 5-III

Biodistribution of liposomes containing neoglycolipid analogs of G_{M1}[1]

Neoglycolipids	111In Distribution (% injected dose)[2]		
	Blood	Spleen	Liver
G_{M1}	52.6 (6.3)	6.6 (0.4)	20.1 (1.1)
DOPE	5.4 (1.3)	11.6 (3.5)	66.6 (8.0)
G_{M1}OS-DOPE	37.1 (1.1)	6.8 (0.8)	41.2 (1.0)
G_{M1}OSNH-X-DOPE	8.0 (0.3)	1.9 (0.3)	77.2 (2.5)
G_{M1}OSNHC8NH-X-DOPE	5.7 (1.2)	9.3 (2.1)	59.2 (1.4)
none	5.9 (1.4)	14.4 (2.0)	67.4 (3.2)

[1]Liposomes composed of egg PC:cholesterol:neoglycolipid (10:5:1) were labeled with ^{111}In-DTPA-SA. 0.2 mg liposomes were injected into each mouse which was sacrificed 3 h after injection.

[2]Data are expressed as mean (S.D.), n=3.

Table 5-IV

Comparison of activities of G_{M1} derivatives in prolonging the liposome circulation, binding to cholera toxin, and inhibiting the complement activation mediated by the alternative pathway.

G_{M1} Derivatives	Relative Effectiveness[1]		
	Prolonging Liposome Circulation[2]	Cholera Toxin Binding	Inhibition of Complement Activation
G_{M1}	+++	+++[4]	+++[7]
Asialo-G_{M1}	+[3]	+[5]	+[7]
Oxidized C7G_{M1}	+	N.D.	N.D.
Reduced C7G_{M1}	+++	+++[4]	+[7]
Hydroxyaminoethyl G_{M1}	+++	+++[4]	N.D.
Methylated G_{M1}	+++	+++[4]	+[7]
G_{M1}OS-DOPE	++	++[6]	N.D.
G_{M1}OSNH-X-DOPE	+	+[6]	N.D.
G_{M1}OSNHC8NH-X-DOPE	+	+[6]	N.D.

[1]Effect of G_{M1} is represented as +++, very good; ++, good; +, poor. N.D., not determined.

[2]Data taken from this study except for asialo GM1.

[3]From Allen and Chonn, (7).

[4]From Schengrund and Ringler (43).

[5]From Holmgren et al. (42).

[6]From Pacuszka et al. (17).

[7]From Michalek et al. (12).

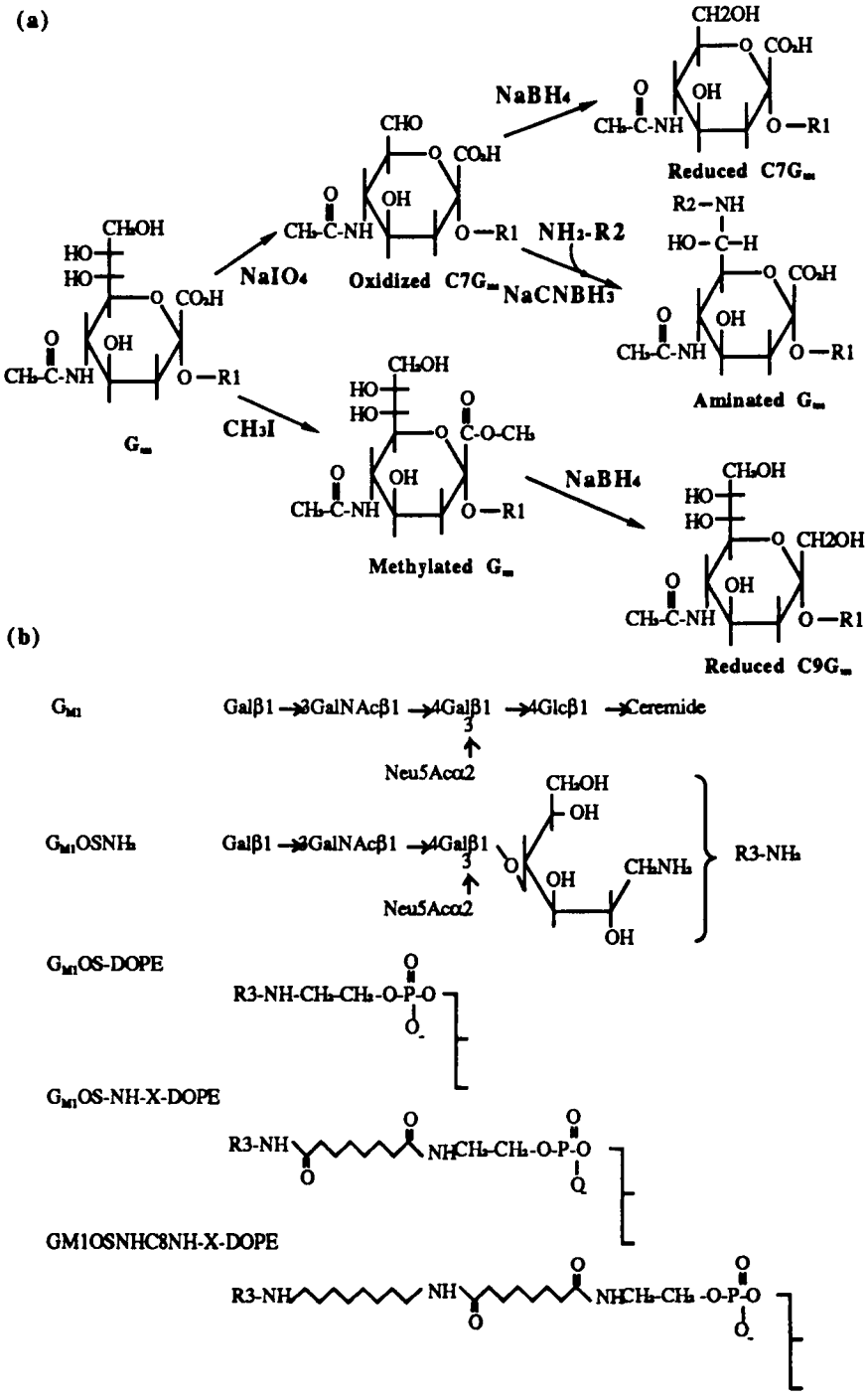

Figure 5-1. Structure of G_{M1} derivatives (a) and neoglycolipid analogs of G_{M1} (b). R1, asialo G_{M1}; R2, aminocompound; R3, oligosaccharide portion of G_{M1} (G_{M1}OS).

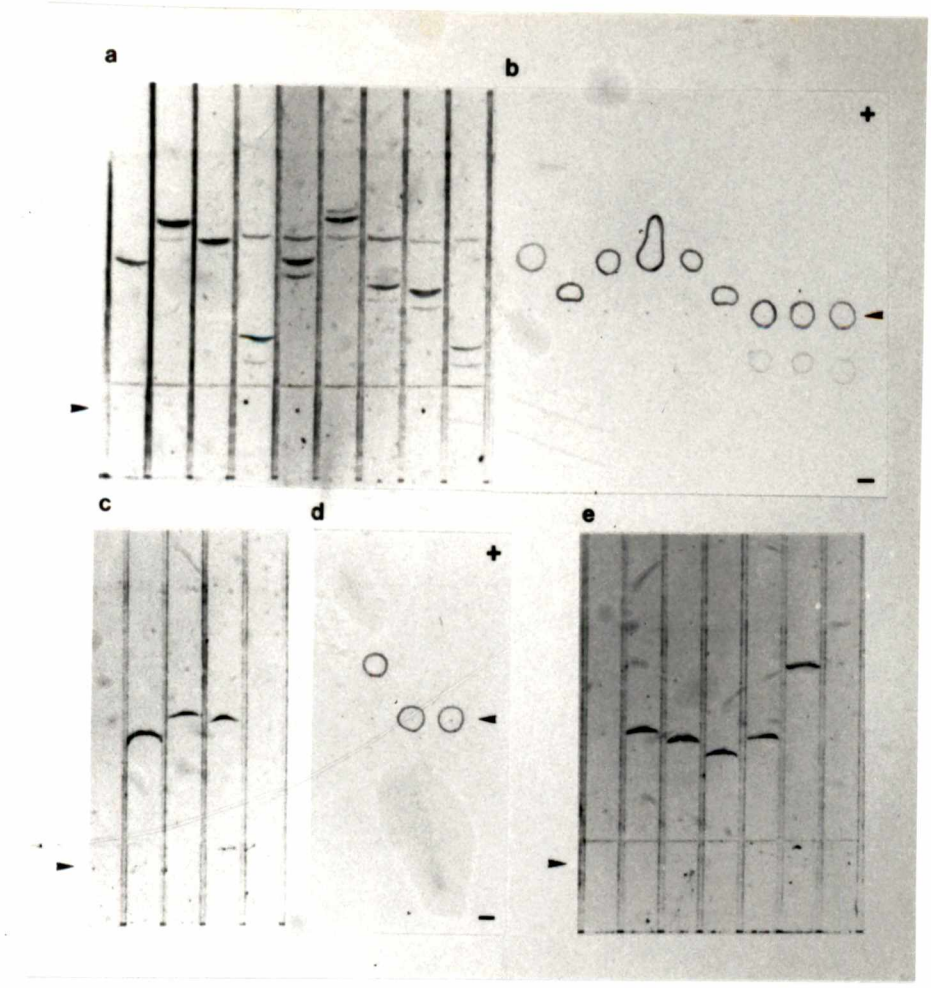

Figure 5-2. Partial characterization of G_{M1} derivatives and neoglycolipid analogs of G_{M1}. (a) Thin-layer chromatograph of G_{M1} derivatives. G_{M1} (lane 1), oxidized $C7G_{M1}$ (lane 2), reduced $C7G_{M1}$ (lane 3), β-alanyl G_{M1} (lane 4), hydroxyethylamino G_{M1} (lane 5), aminoethyl G_{M1} (lane 6), diaminoethyl G_{M1} (lane 7), N-methyldiaminoethyl G_{M1}, N,N-dimethyldiaminoethyl G_{M1} were chromatographed on high performance thin-layer silica gel with the solvent system of chloroform:methanol: 0.2 % $CaCl_2$ (50:45:5) and detected

by resorcinol spray. (b) Paper electrophoresis of G_{M1} derivatives. The G_{M1} derivatives were loaded on Whatman No 1 paper with the same order as in (a), run in methanol/50 mM Tris (pH 8.5) (10:1,v/v) for 30 min at 500 V, and detected by iodine vapor. (c) Thin-layer chromatograph of carboxyl modified G_{M1} derivatives. G_{M1} (lane 1), methylated G_{M1}, and reduced G_{M1} (lane 3) were chromatographed and detected by the same procedure as in (a). (d) Paper electrophoresis of carboxyl modified G_{M1} derivatives. The samples in the same order as in (c) were chromatographed by the same procedure as in (b). (e) Thin-layer chromatograph of neoglycolipid analogs of G_{M1}. G_{M1} (lane 1), G_{M1}OS-DOPE (lane 2), G_{M1}OSNH-X-DOPE (lane 3), G_{M1}OSNHC8NH-X-DOPE (lane 5) and DOPE (lane 4) were chromatographed and detected by the same procedure as in (a).

PART VI

DISTRIBUTION WITHIN THE RETICULOENDOTHELIAL SYSTEM OF LIPOSOMES CONTAINING LIPID A

1. Abstract

Lipid A was incorporated into egg phosphatidylcholine (PC)/ cholesterol liposomes which were tested in mice to investigate its effect on the in vivo biodistribution of liposomes. Addition of lipid A up to 3.2 mol % decreased the hepatic uptake but slightly increased the splenic uptake of the liposomes. When more than 3.2 mol % lipid A incorporated into the liposomes, the hepatic uptake of liposomes increased with the lipid A concentration. Liposomes containing 25 mol % lipid A were rapidly cleared from the circulation and taken up mainly by the liver. Ganglioside G_{M1} or poly(ethyleneglycol)-phosphatidylethanolamine (PEG-PE), which is known to prolong the half life of liposomes in the circulation, was included in the liposome composition along with lipid A. Lipid A antagonized the effect of G_{M1} more effectively than that of PEG-PE. This may be due to the different mechanisms of action exerted by G_{M1} and PEG-PE in the liposome circulation. The hepatic uptake of the liposomes containing lipid A increased with vesicle size. However, the unique splenic accumulation of large PEG-PE liposomes was only slightly affected by the inclusion of a small amount of lipid A (< 7 mol %). These liposomes might be useful for intravenous delivery of antigen to the spleen for an increased immune response.

2. Introduction

Liposomes have been used as carriers of antigens and adjuvants (for review, see ref. 1). A number of laboratories have observed that liposomes containing lipid A can serve as an adjuvant for enhancing the immune responses to many antigens such as cholera toxin, malaria antigens, and other (2-7). Lipid A is the active moiety of lipopolysaccharide (LPS) which is a component in the outer membrane of Gram-negative bacteria and stimulates macrophage activation as well as causing endotoxic shock (for review, see ref. 8). It has recently been suggested that scavenger receptors may be related to the clearance and degradation of lipid A to protect against the effect of endotoxic shock (9,10).

The fatty acid moiety of lipid A is responsible for its endotoxic activities including the Limulus lysate coagulation and pyrogenicity (8). Lipid A activates macrophages to a tumoricidal state by inducing the production of interleukin-1, tumor necrosis factor alpha, and others (for review, see refs. 11-13). These biological activities of lipid A is probably also responsible for its potent adjuvant activity in enhancing the humoral immunity to a wide range of haptens and antigens (2-7). However, because of its potent endotoxicity, lipid A by itself has a limited value as an adjuvant for human vaccine. The endotoxic activities of lipid A can be reduced by incorporation into liposomes (for review, see refs.1,14). However, a controversy exists in the effectiveness of lipid A on macrophage activation when incorporated into liposomes (14).

We have recently reported that liposomes can be formulated to accumulate effectively in the spleen when i.v. injected into mice (15,16). These splenotropic liposomes readily deliver the entrapped antigen to

spleen and are more active in inducing a humoral response to these antigens in the animals than the conventional hepatotropic liposomes (17). Lipid A, which was not used in the formulation of the splenotropic liposomes, could further enhance the adjuvanticity of the liposomes if it does not interfere with the splenic accumulation of these liposomes. Thus we decided to investigate the effect of lipid A on the biodistribution of liposomes.

3. Materials and Methods

a. Materials

Egg PC was purchased from Avanti Polar Lipids (Birmingham, Al). Cholesterol and Lipid A (Salmonella Minnesota) were purchased from Sigma Chemical Co. Ganglioside G_{M1} was purchased from Matreya Inc. (Pleasant Gap, PA). Synthesis of DTPA-SA has been described (18). One μl of $^{111}InCl_3 \cdot HCl$ was mixed with 600 μl of 1 mM DTPA-SA in ethanol at 80 °C with brief sonication. The lack of free ^{111}In in the mixture was confirmed by thin-layer chromatography. PEG-PE (mol. wt. of PEG = 5000) was synthesized as described (16).

b. Liposome preparation

Liposomes were prepared from two parts of egg PC, one part of cholesterol, and various amounts of lipid A with a trace amount of [^{111}In]DTPA-SA which was a nonmetabolizable, nonexchangeable marker of liposomes (19). For the preparation of liposomes containing G_{M1} or PEG-PE, 6.3 mol % G_{M1} or PEG-PE was added to the total lipid composition

consisted of egg PC/cholesterol/lipid A. The lipid mixture was dried under a stream of N_2 gas and vacuum-dessicated for over 1 h to remove any trace of organic solvent. PBS (pH 7.4) was added and the lipid mixture was hydrated for 1 h at room temperature. After rigorous vortexing the suspension was extruded through Nucleopore polycarbonate filters. The extrusion condition was changed depending on the purpose of experiment as follows: large liposomes (over 500 nm in diameter), five times through a 0.8 μm filter; medium sized liposomes (~300 nm), five times through two stacked 0.6 μm filters; small liposomes (~180 nm), ten times through two stacked 0.2 μm filters; very small liposomes (~100 nm), three times through two stacked 0.1 μm filters. Liposome size was measured by a Coulter N4SD submicron particle analyzer (Hialeah. FL).

c. Biodistribution Studies

^{111}In-labeled liposomes (0.2 mg/mouse) were i.v. injected into female ICR mice which were anesthetized with Metofane (Pitman-Moore, NJ) and sacrificed by cervical dislocation. A single three hour time point was chosen for experiments because most liposome samples used in this study had already cleared from the circulation and distributed in tissues. ^{111}In radioactivity of each internal organ was counted with a gamma counter. Weight of mouse blood was assumed to be 7.3 % of the body weight. Blood contamination in each organ was corrected (20).

4. Results

a. Biodistribution of liposomes containing lipid A

To investigate the dependency of the lipid A concentration on the liposome biodistribution, egg PC/cholesterol liposomes (~175 nm in diameter) containing various mol % lipid A were prepared. Biodistribution of these liposomes was analyzed at 3 h post injection. Effect of lipid A showed a biphasic response. Addition of low level of lipid A reduced the liposome uptake by the liver while it slightly increased the uptake by the spleen (Figure 6-1). The hepatic uptake was minimal at 3.2 mol % and then gradually increased with the lipid A mol %. As the hepatic uptake increased, the amount of liposomes in the spleen and blood also decreased. Thus, the biodistribution of liposomes containing lipid A depended on the lipid A concentration. At low density of lipid A, liposomes were more favorably taken up by the spleen with a lower level of hepatic uptake. At higher lipid A content, nearly all liposomes were taken up by the liver: the uptake was very fast, being completed within 15 min of injection (data not shown).

Since liposome localization in organs depends on the vesicle size (21), we have systematically studied the effect of liposome size on the biodistribution of liposomes containing lipid A. Four different size ranges were prepared: very small (~100 nm), small (~180 nm), medium (~300 nm), and large (over 500 nm) liposomes containing no, low (1.7 mol %) or high (25 mol %) content of lipid A. The small and very small liposomes containing 1.7 mol % lipid A showed a lower uptake by the liver than the plain egg PC/cholesterol (2:1, molar ratio) liposomes (Figure 6-2a,b). With increasing vesicle size, the effect of vesicle size subdued the effect of lipid A and the hepatic uptake became dominant and the splenic uptake diminished. However, the biodistribution of liposomes containing 25 mol % lipid A was not affected by the vesicle size; all liposomes were dominantly

taken up by the liver (Figure 6-2c).

b. Lipid A antagonized effect of G_{M1} and PEG-PE on liposome circulation

G_{M1} and PEG-PE show unique effects on prolonging the liposome circulation by suppressing the uptake by RES (22-24). In order to test whether lipid A antagonizes the function of G_{M1} and PEG-PE in prolonging the liposome circulation, various amounts of lipid A were incorporated into egg PC/cholesterol/G_{M1} or egg PC/cholesterol/PEG-PE (10:5:1, molar ratio) liposomes. Liposomes (~180 nm) were i.v. injected into mice and their biodistribution was assayed at 3 h post injection. With increasing mol % of lipid A in the G_{M1}-containing liposomes, the concentration of liposomes in the blood decreased, whereas the hepatic uptake of liposomes concomitantly increased (Figure 6-3). The presence of lipid A at approximately 11 mol % completely abolished the activity of G_{M1} in prolonging the liposome circulation. Unlike the plain egg PC/cholesterol liposomes (Figure 6-1), low density of lipid A in the presence of G_{M1} did not induce any uptake by the spleen. The decreased circulation rate of liposomes was mainly due to increased uptake by the liver.

The effect of lipid A on the circulation of liposomes containing PEG-PE was also studied. Lipid A showed a dose dependent, antagonizing effect on the function of PEG-PE (Figure 6-4a,b). However, its effect on PEG-PE was less pronounced than what was observed for G_{M1} as studied by using liposomes of comparable size distributions, i.e. ~175 nm (Figure 6-4a). Incorporating 11 mol % lipid A only reduced the concentration of liposomes in blood from 60 to 35 % injected dose (Figure 6-4a) whereas the same mol % lipid A in G_{M1}-containing liposomes reduced it to less than 5 % injected dose

(Figure 6-3). Decrease in the blood concentration of liposomes was due to increased hepatic uptake. The uptake by spleen stayed very low for all lipid A concentrations tested.

Vesicle size is one of the critical parameters governing the biodistribution of liposomes containing PEG-PE as well as G_{M1}. It has been reported that large liposomes composed of PEG-PE or G_{M1} (over 300 nm) are preferentially taken up by the spleen (15,16) and do not stay in the circulation for any prolonged period of time. As shown in Figure 6-4b, large liposomes (~420 nm) with low mol % lipid A (below 7.3 mol %) showed a relatively high uptake by the spleen. With the increase of lipid A concentration, the amounts of liposome in the spleen and blood were diminished and the hepatic uptake concomitantly increased (Figure 6-4b). In this case, the effect of lipid A subdued the effect of vesicle size, opposite to what was observed for the plain liposomes containing low amounts of lipid A (Figure 6-2b).

5. Discussion

We have previously reported that large liposomes (d> 300 nm) containing either G_{M1} or PEG-PE accumulate effectively in the spleen (15,16), and these splenotropic liposomes were advantageous in the delivery of antigens for an enhanced humoral response (17). There was an obvious incentive to include lipid A in these liposomes because this bacterial glycolipid has a potent macrophage activation effect which is vital for the stimulation of the immune response. This study was designed to ask the question whether the inclusion of lipid A in the splenotropic liposomes would modify their splenic accumulation after i.v. injection into mice.

The results presented in Figures 3 and 4 clearly indicate that inclusion of lipid A modifies the biodistribution of liposomes. Lipid A effectively antagonized the effects of G_{M1} and PEG-PE and caused liposomes to effectively accumulate in the liver, with little or no splenic accumulation at relatively high lipid A concentrations. Such effect was particularly pronounced on the liposomes containing G_{M1} (Figure 6-3). Even a small amount of lipid A caused a large increase in the liver uptake. When the concentrations of lipid A and G_{M1} were equal (both at approximately 6 mol %), the liposomes behaved just like the conventional hepatotropic liposomes. The antagonizing effect of lipid A was somewhat weaker on PEG-PE (Figure 6-4), but still the liposomes lost a substantial part of resistance to the liver even when small amounts of lipid A was present. The differential sensitivities of G_{M1} and PEG-PE to the effect of lipid A may be related to the difference in mechanism by which the two lipids exert their effect to resist the uptake of liposomes by macrophages. It is well established that PEG-PE provides a strong steric barrier on the liposome surface due to the long, hydrophilic polymer chains. On the other hand, the mechanism of G_{M1} action probably involves more specific recognition by some yet unidentified serum dysopsonin(s) (Park & Huang, unpublished data). It might be easier for lipid A to abolish the specific interaction mediated by G_{M1} than to overcome the strong, nonspecific barrier produced by PEG-PE.

Data shown in Figures 6-1 and 6-2 clearly demonstrated the hepatotropic effect of lipid A. This is consistent with the observation that free lipid A is rapidly cleared from the circulation by the hepatic scavenger receptors (10). Lipid A, being a negatively charged glycolipid, exerts the same charge content to the liposomes. Recent work by Lee et al. (25) has

shown that the uptake of liposomes mediated by the hepatic scavenger receptors requires a high negative charge density of the liposomes. Thus, liposomes containing a high mol % of lipid A may be recognized by the these receptors by the collective aggregation of negative charges on the liposome surface. These results classify lipid A as a strong opsonizing component for liposomes, just like phosphatidylserine and cardiolipin (26,27).

Somewhat unexpected is the observation that liposomes containing no G_{M1} or PEG-PE but a low level of lipid A actually showed a reduced hepatic uptake with concomitant small increase in the splenic uptake (Figure 6-1). The negative charge density of these liposomes are probably not high enough to be recognized by the scavenger receptors (25). However, liposomes containing the same low mol % lipid A but with a larger diameter still effectively taken up by the liver. It is well established that Kupffer cells show higher affinities to larger liposomes (21). Whether such uptake was mediated by the scavenger receptors is not clear.

In summary, the splenotropism of large liposomes containing G_{M1} (15) or PEG-PE (16) is likely reduced by the inclusion of large amounts of lipid A in the liposome membrane. However, data in Figure 4b show that a low level of lipid A (7 mol % or less) in the PEG-PE liposomes did not significantly affect the spleen accumulation of these large liposomes. A low level of lipid A delivered to the splenic macrophages may be sufficient to activate these cells and enhance the immunogenicity of antigen carried by the liposomes. This hypothesis will be tested in future experiments.

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Appendix

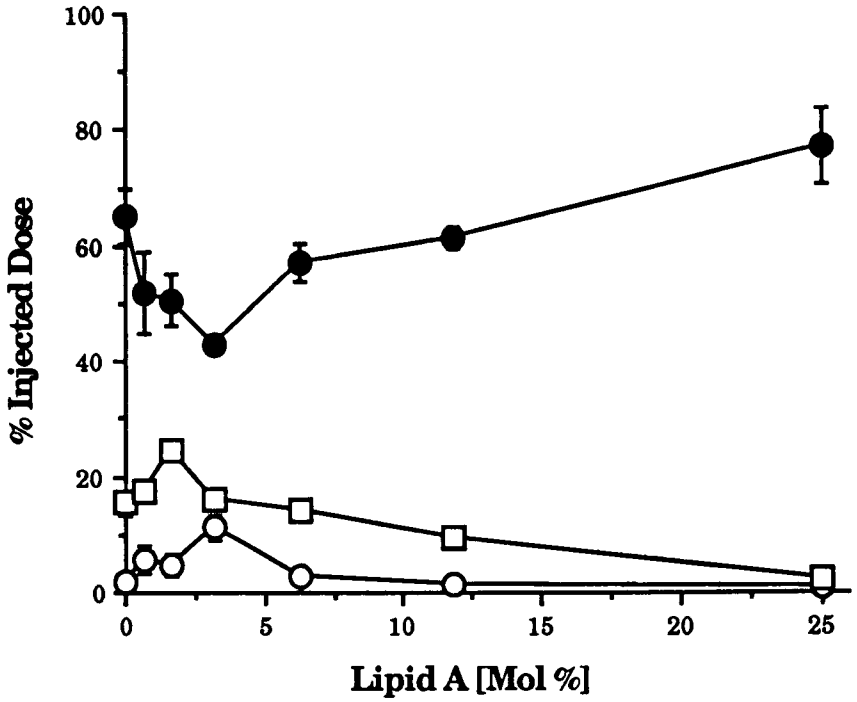

Figure 6-1. Effect of lipid A concentration on the biodistribution of liposomes. Liposomes (~175 nm in diameter) composed of egg PC/cholesterol (2:1, molar ratio) and various amounts of lipid A were labeled with [^{111}In]DTPA-SA. Liposomes (200 μg lipid) were i.v. injected into mice and the radioactivity of blood (○), liver (●), and spleen (❑) was measured at 3 h post injection. Bar represents S.D. (n=3).

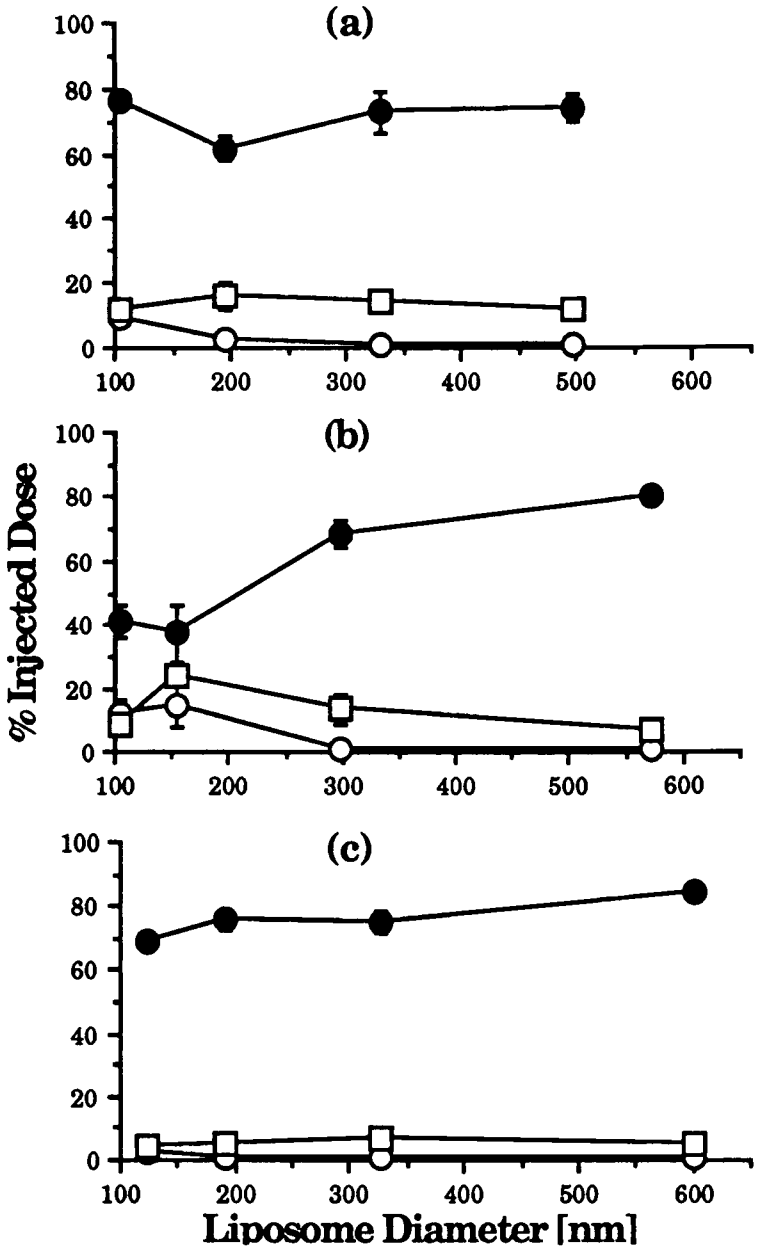

Figure 6-2. Effect of vesicle size on distribution of liposomes containing lipid A in RES. Liposomes composed of egg PC/cholesterol (2:1) and none (a), 1.6 mol % (b), or 25 mol % lipid A (c) were labeled with [^{111}In]DTPA-SA. Liposomes (200 μg lipid) were i.v. injected into mice and the radioactivity of blood (○), liver (●), and spleen (❑) was measured at 3 h post injection. Bar represents S.D. (n=3).

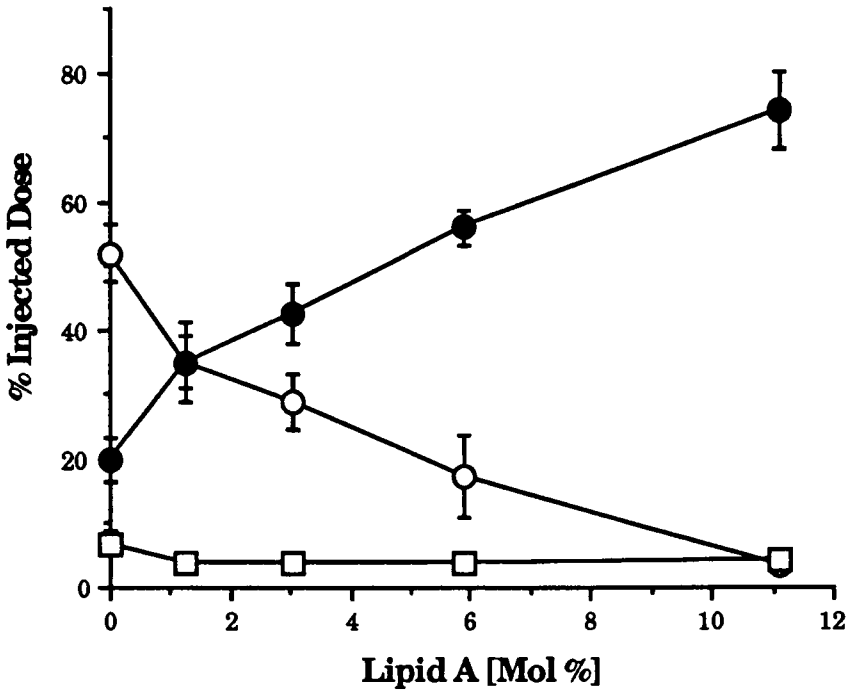

Figure 6-3. Effect of lipid A in antagonizing G_{M1} activity to prolong the liposome circulation. Various amounts of lipid A was incorporated into egg PC/cholesterol/G_{M1} (10:5:1) liposomes (~180 nm in diameter). Liposomes (200 μg lipid) labeled with [^{111}In]DTPA-SA were i.v. injected into mice and the radioactivity of blood (○), liver (●), and spleen (❑) was measured at 3 h post injection. Bar represents S.D. (n=3).

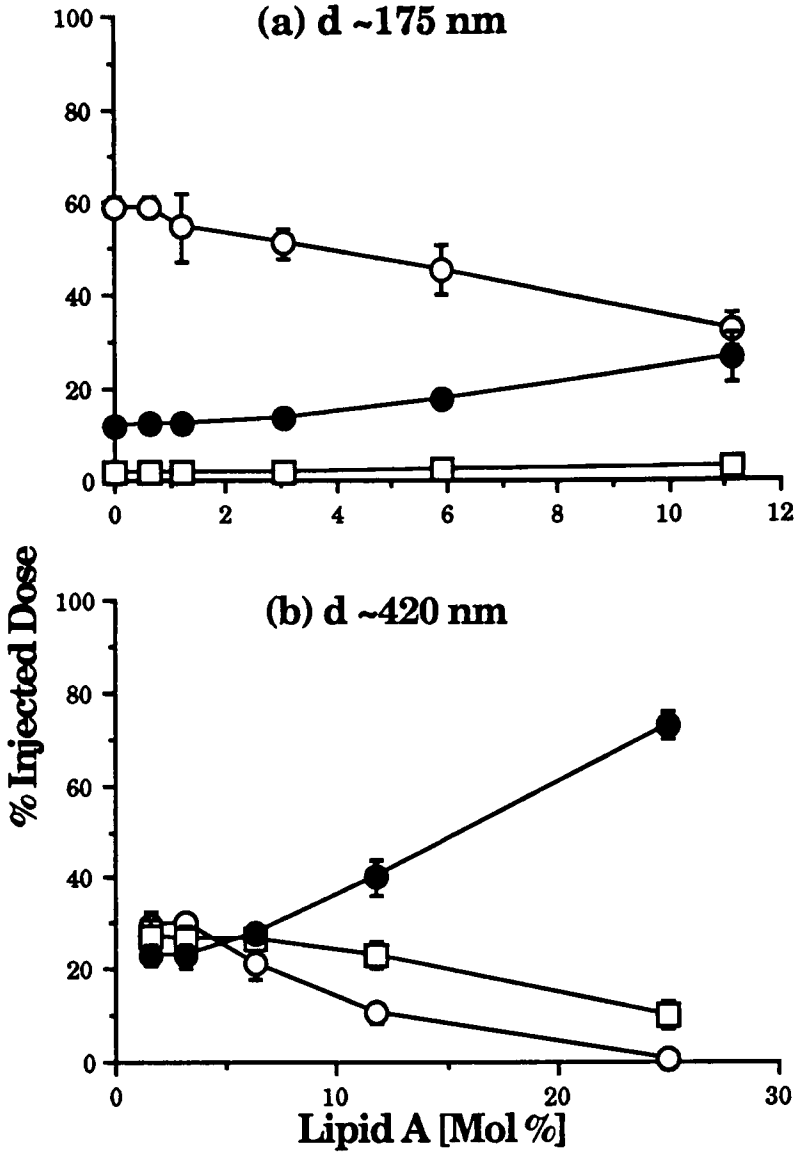

Figure 6-4. Effect of lipid A in antagonizing PEG-PE activity to prolong the liposome circulation. Egg PC/cholesterol/PEG-PE (10:5:1) liposomes with various amounts of lipid A were prepared in two different sizes, i.e. ~175 nm (a) and ~410 nm (b) in diameter. Liposomes (200 μg lipid) labeled with [^{111}In]DTPA-SA were i.v. injected into mice and the radioactivity of blood (○), liver (●), and spleen (❑) was measured at 3 h post injection. Bar represents S.D. (n=3).

PATR VII

SUMMARY AND FUTURE PERSPECTIVES

1. Summary

Coupling of a saccharide unit to the headgroup of DOPE increased the size of lipid headgroup as well as its hydration capability. The synthetic glycophspholipids stabilized DOPE bilayers because they were able to compensate the structural defect of DOPE bilayer. Incorporation of the synthetic glycophospholipid with higher hydration capacity resulted in a larger curvature of vesicles. Especially, the negatively charged trisaccharide-conjugated DOPE (NANL-DOPE) by itself formed micelle (H_I) phase like gangliosides do. NANL-DOPE was also a potent amphiphilic stabilizer for the L_α phase of DEPE bilayer, which is a result of increased interfacial hydration induced by NANL. Another interesting property of NANL-DOPE is its ordering effect on PC bilayers in the L_β phase. The H-bonding network produced by the electronegative sialic acid moiety is probably responsible for this effect.

Some of surface bound carbohydrates are effective in the protection of the liposome membranes from the damage of freeze-thawing or dehydration-rehydration. Trisaccharide-DOPE conjugates showed some cryoprotectant activity, but the mono- and disaccharide-DOPE did not. It has been proposed that the cryoprotective activity of surface bound trisaccharide depends on the H-bonding interaction of the saccharide with the neighboring headgroups of the phospholipids. Several speculations regarding the cryoprotectant effect of surface-bound trisaccharides have been made which includes the extended and flexible saccharide chain, the increased interfacial hydration in the dehydrated state, a steric barrier produced by the bulky sugar group, or diminishing the unstable domain boundaries. It is likely that more than one mechanism is involved in the

cryoprotectant activity of trisaccharide-DOPE. Another interesting observation was that mono- and disaccharide-DOPE are antagonistic to the cryoprotective effect of trehalose. The less flexible and less extended structure of the mono- or disaccharide may interfere with H-bonding of trehalose to the phosphodiester group of the phospholipid.

One of the major roles of the sugar moiety of glycolipid is functioning as a cellular recognition epitope for many biological events. The rate of liposome uptake by the RES is believed to be related to an opsonization process which is also related to the liposomal surface structure, e.g. the presence of glycosyl moiety and the electrical charge distribution. Negatively charged phospholipids are known to enhance liposome uptake by the RES. According to observations shown in Chapter IV, not all negatively charged phospholipids enhance the liposome uptake by the RES; some even reduce the uptake. Among negatively charged DOPE derivatives, only NMPE and NSPE enhanced the liposome uptake. NGPE and NAPE were effective in prolonging the liposome circulation while NPPE and NSBPE were ineffective in either way. On the basis of these observations, a possible mechanism for the action of NGPE and NAPE in the liposome circulation has been suggested in terms of the recognition of hypothetical dysopsonin to the DOPE derivatives.

Some negatively charged glycolipids, e.g. G_{M1}, sulfatides, and PI, prolong the circulation time of liposomes. A putative mechanism for the G_{M1} activity to prolong the circulation time of liposomes is the shielding of the negative charge by a bulky, hydrophilic, and neutral sugar moiety which contributes to the RES avoidance by decreasing or preventing opsonization. However, blocking of the carboxyl group of sialic acid residue hardly affected the G_{M1} activity. Moreover, β-alanyl G_{M1} which had an

additional negative charge showed a comparable activity to the native G_{M1}. These observations imply that shielding the negative charge of sialic acid is not a sufficient mechanism for the action of G_{M1} in prolonging the liposomes circulation time. Oxidized G_{M1} which lost the polyhydroxyl group of sialic acid showed no such activity, but the activity was restored to a different degree by subsequent reduction or coupling of amino compounds under reducing condition. Also, G_{M1}OS directly attached to DOPE was effective in prolonging the circulation time of liposomes while G_{M1}OS attached to DOPE via spacer arms was not. This structure-activity study has also revealed a striking paralleled correlation with the binding of cholera toxin to G_{M1}. Taken together, the data suggest that a cholera toxin like dysopsonin(s) is responsible for the activity of G_{M1} to resist the RES uptake of liposomes. Binding of the hypothetical G_{M1}-specific dysopsonin(s) to liposomes could lead to a reduced level of opsonization and/or inactivation of bound opsonin(s).

Another glycolipid studied in this project is lipid A which is the lipid portion of lipopolysaccharides of Gram-negative bacteria. Lipid A has been used in immunotherapeutic applications because it is able to activate macrophages to a tumoricidal state and enhance the humoral immunity. Clearance rate and in vivo deposition of liposomes containing lipid A was dependent on the mol % lipid A. High mol % lipid A enhanced clearance rate and hepatic uptake of liposomes, while low mol % lipid A affected little the clearance rate, but reduced the hepatic uptake. Lipid A antagonized the G_{M1} activity more effectively than PEG-PE in terms of the liposome circulation time. This is probably due to the difference in the functional mechanism by which G_{M1} and PEG-PE prolong the liposome circulation time.

2. Future Perspectives

The biophysical studies described in this thesis provide some general understandings of the physical behavior of a series of glycolipids. However, it was not possible to reveal any detail information regarding the structure and interactions of glycolipid in a complex lipid mixture or in membranes. Therefore, future spectroscopic studies are needed to substantiate the unexpected observations, e.g. NANL-DOPE effect on the membrane fluidity in L_β phase. ^2H NMR studies (1) of NANL-DOPE labeled with ^2H in specific positions of the acyl chain or of the head group should provide useful information regarding the membrane fluidity in the L_β and L_α phase. Studies of the solution conformation of the glycosyl headgroup of glycophospholipids using ^1H-NMR (2) and X-ray diffraction (3) would also be helpful to understand the configuration of glycosyl headgroup on the membrane surface of phospholipid and its interaction with neighboring headgroups of the phospholipids.

The cryoprotectant ability of surface bound trisaccharides was not strong enough for a liposome preparation which is stable in the frozen or dehydrated state. In order to develop cryoprotecting glycolipids as strong as free trehalose in solution, several strategies may be employed. First of all, trehalose could be conjugated to a lipid anchor via a spacer arm of suitable length. Trehalose has been conjugated to other macromolecules, such as proteins, for different purposes (4). In addition, the lipid anchor, DOPE, could be substituted by other lipids, such as saturated phospholipids or nonphospholipids. The cisdouble bonds in the acyl chains of DOPE may affect the bilayer stability during the freeze-thaw cycles.

One of the major problems in the area of drug delivery using liposomes as a carrier is the rapid clearance and RES uptake of liposomes. A great deal of effort has been spent to develop liposomal formulations to enhance the circulation time as well as reducing the RES uptake. Even though the recent outstanding advances regarding the liposomes with prolonged circulation (5-8) have provided new opportunities for the liposomal drug delivery, we are still far away from utilizing liposomal systems in clinical medicine. It is difficult to promote the systems to a higher stage of usage without knowing the mechanism of liposome clearance and uptake by the RES cells. Since the opsonization process has been demonstrated for the liposome uptake by macrophages, an urgent study will be the identification and characterization of opsonic component(s) in the blood. Identification of dysopsonic component(s) (9) is equally important because the elucidation of its mechanism of action will certainly bring about revolutionary advances to overcome many obstacles now existing. Development of improved formulations for long circulating liposomes will certainly expand the role of liposomes in numerous potential clinical applications.

In addition to the prolonged circulation time, efficient targeting to the desired tissues or cells and transferring the entrapped drugs to the target cell are desirable characteristics of an ideal drug carrier. The pH-sensitive liposomes are useful only for cells which take up liposomes via endocytosis. Also, the development of long circulating liposomes will solve only the half of the problem regarding the targetability of liposomes. It has been shown that long circulating pH-sensitive immunoliposomes show relatively poor target-binding affinity (10). The reduced binding affinity of antibody might be improved by a different method of antibody conjugation. For cells which

do not actively endocytose liposomes, fusogenic immunoliposomes, which are able to circulate long enough for targeting, will be the best solution. Developing formulations for stable, but selectively fusogenic liposomes with certain fusogenic materials, i.e. fusogenic lipids and fusogenic proteins, will be an ultimate contribution in the field of liposomal drug delivery.

Because of its potent endotoxic activities, application of liposomes containing lipid A as an adjuvant for use in human vaccines needs very careful optimization for safety as well as efficacy. It has been suggested that scavenger receptors may be related to the clearance and degradation of lipid A to protect against the endotoxic shock effect (11,12). Therefore, it is necessary to investigate whether liposomes containing lipid A is also recognized and processed by the same receptors. Ligands for the scavenger receptors, e.g. maleylated BSA, acetylated LDL, polyinosinic acid, etc., effectively block specific biding and uptake of lipid A. These ligands could be used to block the uptake of liposomes containing lipid A if scavenger receptors are responsible for their rapid uptake by the liver. Recently developed monophosphoryl lipid A (by Ribi Immunochem, Inc.) which is less toxic but equally active as a macrophage activator as the native lipid A , should be tested in the liposomes formulation. It would be interesting to see if scavenger receptors can recognize this lipid A derivative and, if so, how does it modify the clearance and biodistribution of liposomes.

3. References

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VITA

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