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Liposomes and bronchoalveolar lavage fluid: Release of vesicle-entrapped glutathione

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Summary

Interest in the potential applications of liposomes for pulmonary drug delivery prompted this in vitro study of the effect of lipid composition on vesicle permeability. Rat cell-free bronchoalveolar lavage fluid was incubated at 37°C with large unilamellar liposomes, prepared by the extrusion technique, containing entrapped [^{3}H]glutathione (GSH). Lavage-induced GSH leakage occurred for liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) However, leakage from dimyristoylphosphatidylcholine (DMPC) liposomes in the presence of lavage was not different from leakage in control buffer. Cholesterol was found to enhance GSH release from distearoylphosphatidylcholine (DSPC) and DPPC vesicles, and to stabilize DMPC vesicles. The results suggest that DPPC liposomes may be more suitable than vesicles prepared from DSPC or DMPC for effective delivery of GSH to the lung, and that variation of cholesterol content can be used to enhance selectively or diminish the in-vitro release characteristics

Introduction

The use of liposomes to achieve organ-specific or target-specific delivery of therapeutic, prophylactic or diagnostic agents is of considerable current interest. We have applied liposome technology in our research with the objective towards improving pulmonary delivery. In the literature, liposomal delivery to the lung has been investigated by two general approaches, namely systemic delivery (Hunt et al., 1979; Zachman and Tsao, 1980; Abra et al., 1984) and the more direct route of intratracheal (Juliano and McCullough, 1980; Oyarzum et al., 1980; Pettenazzo et al., 1989) or aerosol delivery (Ivey et al., 1980, Wyde et al., 1988). Successful delivery by the former approach is dependent on sustaining adequate drug availability in the lung after liposomes have undergone systemic dilution, selective uptake by the reticuloendothelial system, and possible degradation in the bloodstream (Yatvin and

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Lelkes, 1982; Bonte and Juliano, 1986). With the latter approach, assuming successful direct delivery to the lung, drug availability is dependent on drug release from the liposomal capsules. The process of aerosolization, however, can cause drug release to varying degrees before the introduction of liposomes into the lung (Niven and Schreier, 1990; Taylor et al., 1990a). In vivo determination of therapeutic efficacy provides an indication of drug availability, and therefore, presumably of drug release. It is easier, however, to study drug release from liposomes in vitro. Liposomes delivered into the lungs will first come in contact with the bronchoalveolar fluid. This fluid contains several components, such as albumin and immunoglobulins, which are also found in serum (Bell et al., 1981; Henderson, 1988). Serum factors have been shown to affect liposome stability (Allen, 1981). To date, the effect of bronchoalveolar lavage fluid (BAL) on liposome stability has not been examined.

In this report, we described the leakage characteristics of liposomes, entrapped with reduced glutathione (GSH), in the presence of rat BAL at 37°C. The water-soluble tripeptide, GSH, was chosen as the agent for liposomal encapsulation because we are interested in its potential protective role against free radical-mediated injuries in the lung. In a previous investigation, we demonstrated enhanced GSH retention in the rat lung after its intratracheal instillation in liposome-encapsulated form (Jurima-Romet et al., 1990). In this study, we used large unilamellar vesicles (LUV) prepared by the extrusion technique (Hope et al., 1985). This method yields a homogeneous population of vesicles of a selected diameter. Since vesicles greater than about 0.5 μm in diameter do not seem to have a good alveolar distribution in the rat lung (Belbeck, L., personal communication), we prepared liposomes of less than 0.5 μm for delivery to the rat in our experiments. Our previous study has demonstrated that the entrapment efficiency for GSH is approx. 20% using LUV (Jurima-Romet et al., 1990); other liposomal preparations such as multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) are well known to have much lower entrapment efficiencies.

Both the entrapment of water-soluble compounds within liposomes and their leakage from the vesicles are dependent, to a large degree, on the lipid composition of the carrier vesicle. Near their gel-to-liquid-crystalline phase transition temperature (T_c), the permeability of liposomes to water-soluble agents is enhanced (Szoka and Papahadjopoulos, 1980). Cholesterol also plays an important role in the biophysical and biochemical properties of liposomes, affecting their fluidity, permeability and the organization of their lipid bilayers. Both the phospholipid and cholesterol components of liposomes influence their metabolic turnover in and interaction with cells (Poste and Papahadjopoulos, 1978). In our previous in vivo study in the rat (Jurima-Romet et al., 1990), we used liposomes composed of egg phosphatidylcholine (PC), cholesterol and stearylamine. However, egg PC is a heterogeneous mixture of phospholipids of undefined chain lengths. In order to investigate the role of phospholipid composition on liposome stability, pure synthetic phospholipids of defined chain length are required. Therefore, in this study, we used three synthetic phospholipids with saturated long-chain fatty acids: distearoylphosphatidylcholine (DSPC; 18-carbon chains; $T_c = 55°C$), dipalmitoylphosphatidylcholine (DPPC; 16-carbon chains; $T_c = 41°C$), and dimyristoylphosphatidylcholine (DMPC; 14-carbon chains; $T_c = 23°C$), for preparing different liposomal formulations. For this series of homologous phosphatidylcholines, we also examined the effect of cholesterol on GSH leakage from liposomes in the presence of BAL. Our results may be of benefit for the future design of liposome-entrapped drugs or agents for pulmonary delivery.

Materials and Methods

Chemicals

Egg yolk PC, DMPC, DPPC, DSPC, cholesterol, stearylamine, GSH and Ellman's reagent (5,5,-dithiobis[2-nitrobenzoic acid]) were obtained from Sigma (St. Louis, MO). [^{3}H]GSH, Protosol tissue solubilizer and liquid scintillation cocktails were purchased from New England Nuclear (Bos-

ton, MA). [^{14}C]Cholesterol was obtained from Amersham (Arlington Heights, IL). All reagents and solvents were of analytical grade.

Animals

Male Wistar rats, 220–280 g in weight, were purchased from Charles River Canada, Inc. (St. Constant, Quebec). Animals used in this research were cared for in accordance with the principles contained in the *Guide to the Care and Use of Experimental Animals*, prepared by the Canadian Council on Animal Care.

Pulmonary lavage procedure

Rats were anaesthetised with sodium pentobarbital (50 mg/kg i.p.) and the trachea cannulated with PE-200 polyethylene tubing. A 16-gauge needle with the point cut off was inserted into the cannula. The other end of the needle was attached via a three-way stopcock to two 20-ml syringes. Phosphate-buffered saline (PBS), consisting of 0.15 M sodium chloride/0.01 M sodium phosphate (pH 7.4), was prewarmed to 37°C and instilled into the lung via one syringe and lavage fluid withdrawn via the other. The lavage procedure was repeated 10 times, using 5 ml of PBS each time. The rate of instillation was about 10 s per wash.

The BAL was spun at $1000 \times g$ for 10 min at 5°C to pellet bronchoalveolar cells. The supernatant was removed and concentrated to a volume of approx. 4 ml by using a 50 ml Amicon filtration vessel with a PM-10 membrane filter. Protein concentration was determined by the Bio-Rad assay (Bradford, 1976) using bovine serum albumin as a standard. The concentrated BAL supernatant was divided into aliquots and stored at −80°C until leakage assays were performed at which time they were diluted with PBS (pH 7.4) to the desired protein concentration.

Preparation of liposomes

A total of 100 mg of lipid was dissolved in chloroform/methanol (2:1, v/v) and 0.3 μCi of [^{14}C]cholesterol was added as a tracer to label the lipid component of the liposomes. The lipid was dried onto the sides of the test tube under a stream of nitrogen. Residual solvent was removed by placing the test tube under vacuum for at least 1 h. To the dried lipid, 10 μCi of [^{3}H]GSH and 1 ml of 0.4 M GSH in 5 mM potassium phosphate buffer (pH 6.5) containing 30 mM EDTA were added. In order to form liposomes, it is necessary to hydrate the lipid at a temperature higher than the T_c of the lipid. Therefore, liposomes composed of DSPC, DPPC, and DMPC were hydrated with the GSH solution at 65, 51 and 33°C, respectively, i.e., 10°C above the T_c of the lipid. The stability of GSH at these elevated temperatures was confirmed by determining that no loss of sulfhydryl group reactivity occurred during heating of GSH solution at 100°C for 1 h. MLV were formed during vortexing of the lipid-aqueous mixture. The MLV were then frozen in liquid nitrogen and thawed in a warm waterbath, the freeze-thaw cycle being repeated five times (Hope et al., 1985). The preparation was then extruded, under helium, 10 times through two stacked polycarbonate filters (Nucleopore) of 400 nm pore size using an Extruder (Lipex Biomolecules Inc., Vancouver, B.C.). To maintain the liposome preparation at 10°C above T_c during extrusion, the Extruder was fitted with a thermal barrel connected to a temperature-controlled circulating waterbath.

After extrusion, the liposomes were kept at 5°C. Non-entrapped GSH was removed by washing the liposomes twice in 5 mM potassium phosphate buffer (pH 6.5) and centrifuging at 110 000 $\times g$ for 1 h at 5°C. The entrapment efficiency for GSH was determined by counting ^{3}H and ^{14}C concentrations in aliquots of supernatants and pellets using a Beckman LS-5801 liquid scintillation spectrometer. The recovery of ^{14}C in the final pellet was always greater than 85%. The final pellet was resuspended in 6 ml of buffer at 5°C and used immediately. Initial experiments examining the chemical stability of liposomal GSH upon storage and the effect of BAL dilution were carried out with vesicles composed of egg phosphatidylcholine, cholesterol and stearylamine in a molar ratio of 6:3:1, as in our previous study (Jurima-Romet et al., 1990). For the subsequent study of GSH release with BAL, liposomes were prepared from DSPC, DPPC or DMPC, each with or without cholesterol (15 and 30 mol%).

GSH leakage assay

Washed liposomes, approx. 2 ml, were mixed with an equal volume of pulmonary lavage and incubated in capped test-tubes at 37°C. Control incubations at 37°C consisted of liposomes mixed with an equal volume of PBS (pH 7.4). Time zero samples were prepared by mixing equal volumes of liposomes with BAL or buffer at 5°C. At specified times, 0.2-ml aliquots in duplicate were removed from the incubation mixtures and transferred to Beckman 7 × 20 mm cellulose propionate ultracentrifuge tubes. The tubes were ultracentrifuged at 130 000 × g for 40 min at 5°C.

The supernatant was removed as completely as possible and transferred to a scintillation vial. Aquasol was added and ^{3}H and ^{14}C radioactivity determined by liquid scintillation counting. The ultracentrifuge tube containing the pellet was placed in a glass scintillation vial and 0.3 ml of Protosol tissue solubilizer : ethanol, 1 : 2 (v/v), was added to the ultracentrifuge tube using a Pasteur pipet. The vial was allowed to stand for 2–3 h or until the pellet was completely dissolved. For liposomes prepared from DSPC, it was necessary to heat the vials at 45°C for approx. 1 h to dissolve the pellet. Biofluor was added to the solubilized pellet and the radioactivity measured by liquid scintillation counting.

Supernatant ^{3}H counts represented the leakage of GSH from liposomes; GSH latency was expressed as ^{3}H counts in the pellet as a percentage of total ^{3}H counts in the pellet plus supernatant. Supernatant ^{14}C counts were near background levels for all samples, indicating that the liposome-containing pellet was not disrupted during transfer of the supernatant.

The unpaired Student's t-test was used to compare GSH leakage in the presence of BAL to leakage in PBS controls.

Vesicle size analysis

Quasi-elastic light scattering was used to determine the size distribution of liposome preparations. A Coulter N4SD particle-size analyzer was employed. A small aliquot of the sample was diluted to about 2 ml with PBS (pH 7.4) and light scattering recorded at 20°C. Unimodal analysis was performed and the method of cumulants used for analysis of the autocorrelation functions.

GSH determination

The chemical stability of entrapped GSH during storage at 5°C was determined by the colorimetric Ellman assay (Ellman, 1959). A 0.1 ml aliquot of liposomes and 0.1 ml of Ellman's reagent were added to 5 ml of 0.1 M sodium

TABLE 1

Glutathione entrapment efficiency and vesicle size of liposomes prepared from three different phosphatidylcholines with and without cholesterol

Lipid composition of liposomes		GSH entrapment efficiency (%)	Mean vesicle diameter (nm)	Effect of cholesterol p
PL	CSL			
DSPC	–	21.0, 20 6	416 ± 56 (6)	–
DSPC	15%	18.9, 19 6	299 ± 02 (3)	< 0 05
DSPC	30%	17.3, 18 1	270 ± 09 (5)	< 0 001
DPPC	–	13 8 ± 1 9 (4)	317 ± 39 (3)	–
DPPC	15%	10 3 ± 1 4 (4)	203 ± 06 (6)	< 0.001
DPPC	30%	16 3 ± 2 0 (3)	177 ± 07 (5)	< 0.001
DMPC	30%	16 1 ± 1 6 (5)	243 ± 03 (3)	n d

The experiments were carried out as described in Materials and Methods. Numbers in parentheses indicate the number of individual determinations and S E is reported where n = 3–6 For each type of phosphatidylcholine, the probability of a significant effect of cholesterol at 15 and 30 mol% on the mean vesicle diameter was determined by unpaired Student's t-tests. CSL, cholesterol; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine, PL, phospholipid; n.d., not determined.

phosphate buffer (pH 8.0) containing 1% Triton X-100. The liposomes lysed in the presence of Triton and released the entrapped GSH. The absorbance at 412 nm was measured using a Bausch and Lomb 1001 Spectronic spectrophotometer and compared to freshly prepared standards of known GSH concentrations.

Results

GSH entrapment efficiency and vesicle size

The GSH entrapment efficiency for liposomes prepared from DSPC and DPPC ranged from 8 to 21% (Table 1). The incorporation of cholesterol had negligible effect on GSH entrapment for liposomes composed of these phospholipids. For liposomes prepared from DMPC, the entrapment efficiency was only about 3% and therefore, this phospholipid was not used in the BAL leakage study. However, the entrapment of GSH into DMPC vesicles was increased to about 16% by the incorporation of cholesterol at 30 mol%. A lower concentration of cholesterol at 15 mol% did not improve the poor GSH entrapment for DMPC liposomes.

Although liposomes of all lipid compositions were extruded through filters of the same pore size (400 nm), there was considerable variation in their size distribution (Table 1). The mean diameter was largest for vesicles prepared from DSPC with no cholesterol. The incorporation of cholesterol in DSPC and DPPC liposomes significantly reduced the average vesicle diameter of the distribution.

Chemical stability of GSH

Since GSH is readily oxidized, its stability within liposomes was investigated under conditions which lessen the potential for oxidation: EDTA was added to the buffer used for preparing GSH solutions and liposome preparations were stored under inert gas. It can be seen from Table 2 that failure to take these precautions resulted in a relatively rapid and substantial oxidation of GSH. When liposome-entrapped GSH was left in buffer exposed to atmospheric air, only 68% of non-oxidized GSH remained after 1 day. Subsequent loss in GSH activity was less rapid. After 14 days, 45% of non-oxidized GSH remained. In contrast, the formulation and storage of liposome-encapsulated GSH in an EDTA-containing buffer, under an inert atmosphere of helium, was found effective in preserving practically all the GSH activity for at least 2 weeks. Therefore, the latter condition was used to maintain the chemical stability of liposomal GSH for all leakage experiments conducted in this study.

TABLE 2

Chemical stability of GSH in liposomes upon storage

Storage period (day)	% residual non-oxidized GSH	
	Air	Helium
1	68	94
7	58	105
14	45	107

Large unilamellar vesicles prepared by the extrusion technique, as described in Materials and Methods, were used These liposomes were composed of egg phosphatidylcholine, cholesterol and stearylamine in a molar ratio of 6.3.1 The buffer was 5 mM potassium phosphate (pH 6.5) and the liposomes were stored at 5°C in the buffer under an atmosphere of air or in the same buffer containing 30 mM EDTA under helium for different time periods The residual non-oxidized glutathione concentration was measured at the end of each storage period.

No oxidation of GSH occurred during liposome formation; the entrapment efficiency, determined by measurement of sulfhydryl groups, was found to be identical to the value obtained by radioactivity measurement.

Effect of dilution of pulmonary lavage on GSH leakage

The dilution of the lavage fluid, adjusted according to different protein concentrations as a convenient reference, was found to affect GSH release from liposomes (Fig. 1). Incubation with BAL having a protein content of 100 or 75 $\mu g/ml$ resulted in significantly greater leakage than incubation with lavage of 25 $\mu g/ml$ protein concentration. Leakage was also greater at protein contents of 100 or 75 $\mu g/ml$ in comparison to 50 $\mu g/ml$, although the differences were not statistically significant at all time points. There were no

significant differences between the 100 and 75 μg/ml leakage curves, suggesting that BAL-induced release of GSH approaches a maximum at a protein concentration of about 75 μg/ml of protein. Therefore, for all subsequent studies investigating the effect of liposomal lipid composition on GSH leakage, lavage samples were standardized to 75 μg/ml of protein.

Effects of cholesterol and fatty-acid chain length of the phospholipid on GSH leakage

[^{3}H]GSH leakage experiments demonstrated that, in the presence of rat BAL, the 18-carbon chain-length DSPC liposomes containing no cholesterol or 15 mol% cholesterol retained 85% of entrapped GSH after 72 h of incubation at 37°C (Fig. 2). These results were not significantly different from control incubations of DSPC liposomes in PBS. However, cholesterol at 30 mol% produced significantly enhanced gradual release; 65% of the GSH remained in the liposomes after 72 h ($p < 0.05$).

The 16-carbon chain length DPPC liposomes without cholesterol were fairly resistant to BAL-induced leakiness during the first 24 h of incubation (Fig. 2). This initial stability was followed by a more rapid loss of GSH latency such that by 72 h, only 27% of the tripeptide remained entrapped

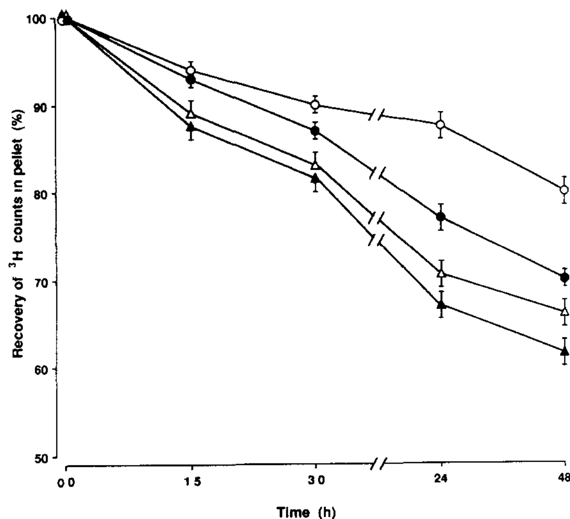

Fig. 1. Effect of dilution of pulmonary lavage, as a function of protein concentrations, on GSH leakage from liposomes. Liposomes were prepared from egg phosphatidylcholine, cholesterol and stearylamine (6 3 1 molar ratio) and contained entrapped [^{3}H]GSH. The vesicles were incubated at 37°C with cell-free bronchoalveolar lavage fluid having protein concentrations of 25 μg/ml (○), 50 μg/ml (●), 75 μg/ml (△), and 100 μg/ml (▲). Data represent mean ± S E. of lavage fluids from 3–6 animals and were obtained using two separate liposomal preparations

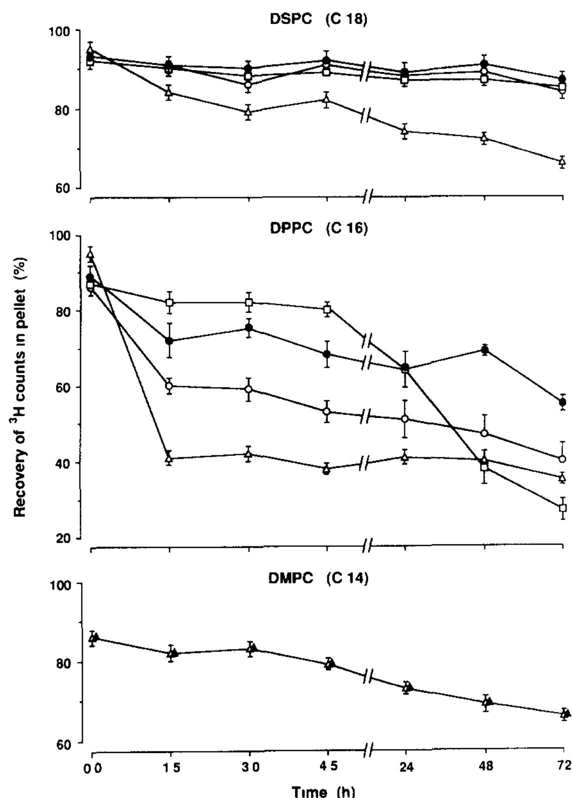

Fig 2 Effects of cholesterol content and phospholipid fatty-acid chain length on GSH leakage from liposomes incubated with pulmonary lavage fluid Liposomes composed of DSPC, DPPC, or DMPC, and either 0% cholesterol (□), 15% cholesterol (○) or 30% cholesterol (△), and containing entrapped [^{3}H]GSH were incubated at 37°C in phosphate-buffered saline (PBS, solid symbols) or cell-free bronchoalveolar lavage (open symbols) For DSPC and DPPC, the PBS data are plotted on single curves, since there were no significant differences between individual curves obtained with liposomes containing 0, 15 and 30% cholesterol. Lavage data represent mean ± S E of lavage fluids from 4–6 animals and were obtained using at least two separate liposomal preparations

within the lipid vesicles. The leakage in BAL at 48 h and 72 h was significantly greater than leakage in PBS controls ($p < 0.05$). The effect of cholesterol on DPPC liposomes was to produce earlier and more pronounced losses of GSH, which were significantly different from the PBS controls ($p < 0.05$). Thus, for liposomes containing 15 mol% cholesterol, only 60% of GSH remained encapsulated at 1.5 h. For liposomes containing 30 mol% cholesterol, GSH latency was even lower, that is, 42% at 1.5 h. The apparent first-order rate constant for the release of GSH at 1.5 h was 1.07 h^{-1} for DPPC vesicles without cholesterol, 2.20 h^{-1} for DPPC vesicles containing 15 mol% cholesterol, and 2.65 h^{-1} for DPPC vesicles containing 30 mol% cholesterol. However, after 72 h of incubation, the amount of GSH which remained liposomally-entrapped was similar for all DPPC preparations, and significantly less than for PBS controls ($p < 0.05$).

The observations with DMPC (14-carbon chain length) liposomes were complicated by the poor GSH entrapment efficiency for this phospholipid in the absence of cholesterol or with 15 mol% cholesterol. Meaningful data on GSH leakage could be obtained only with DMPC liposomes containing 30 mol% cholesterol (Fig. 2). These vesicles demonstrated a gradual leakage of GSH from 86% latency at time zero to 66% at 72h. However, since no significant differences were observed between incubations with PBS and incubations with BAL, it appears that leakage from DMPC vesicles containing 30 mol% cholesterol is not related to BAL but rather, is a time and/or temperature-related occurrence.

Discussion

The results of this study demonstrated that cell-free rat BAL mediated the release of GSH from liposomes composed of DSPC or DPPC. The identity of the factor in BAL responsible for GSH release is unknown. Since BAL contains a variety of proteins, it is possible that a protein factor may be involved. The majority of proteins in BAL are similar to those found in plasma (Bell et al., 1981). Proteins present in the BAL from dogs have been reported to be 72% albumin and 8% IgG (Henderson, 1988). The interactions of liposomes with serum proteins have been studied. Adsorption of serum albumin to the outer surface of liposomes has been shown to take place. This interaction, however, does not seem to affect the integrity of the vesicles (Bonte and Juliano, 1986). On the other hand, the interactions between serum immunoglobulins and liposomes are complex and may influence vesicle stability (Wolff and Gregoriadis, 1984).

Liposomal destabilization in the presence of blood or serum has been mainly attributed to the removal of phospholipid from the bilayers by high-density lipoprotein (HDL) (Chobanian et al., 1979; Allen, 1981). However, lipoprotein was reported to be absent from BAL obtained from healthy human subjects (Bell et al., 1981). Fractionation of rat BAL, by high-performance liquid chromatography, revealed the presence of nine protein components, three of which were common to BAL and blood (albumin, immunoglobulins) while the others were unique to the lung (Lehnert et al., 1986). Surfactant-associated proteins, which include a highly glycosylated protein of molecular mass about 35 kDa and lipophilic proteins of low molecular mass, can dramatically alter the surface tension of phospholipids (Jobe and Ikegami, 1987). It is possible that rat BAL-mediated release of GSH is attributable to immunoglobulins or surfactant-associated proteins. Our observation of a two-stage pattern of GSH release from DPPC liposomes, i.e., a rapid initial release followed by a slower sustained release, is similar to observations by Hunt (1982) on the interaction of liposomes with plasma. Hunt suggested that the release kinetics may be explained by an interaction of membrane lipids with plasma proteins. In our investigation, another possibility is that lipids present in BAL may have contributed to GSH release.

Although serum usually destabilizes liposomes, sometimes a stabilizing effect occurs: Lelkes and Friedmann (1984) demonstrated a reduction in carboxyfluorescein release from DPPC vesicles in the presence of serum, incubated at a temperature below T_c. This reduction was further augmented by the incorporation of cholesterol into

the lipid bilayer. These observations contrast with our present observations regarding BAL where cholesterol was found to have a destabilizing effect on DPPC vesicles.

The concentration of liposomal lipid in our incubations with BAL was selected to approximate amounts that have been used for in vivo delivery of liposomes to rats by intratracheal instillation (McCullough and Juliano, 1979; Jurima-Romet et al., 1990). At a protein content of 75 μg/ml, the BAL fluid was concentrated to about half the volume of fluid present in the rat lung (Henderson, 1988); after a 1 : 1 dilution with liposomes, it approximated the fluid volume of the rat lung. Therefore, the relative concentrations of liposomes and BAL in our in vitro experiments were selected to simulate in vivo conditions.

In the present study, we only examined the effect of cell-free pulmonary fluid on the stability of liposomes. Therefore, the possible contribution of the cellular component of the lung milieu was not addressed. However, it is well known that alveolar macrophages have an important role in the removal of various inspired particles that deposit in the lung (Weinstock and Brain, 1988). Generally, greater than 90% of free cells recovered from pulmonary lavage are alveolar macrophages (Brain and Frank, 1968). The ability of lung lavage cells to phagocytose liposomes has been reported (Shephard et al., 1981). Other cell types, for example, type II alveolar epithelial cells, also take up liposomal phosphatidylcholines (Zachman and Tsao, 1980).

In the design of liposomes for drug delivery to increase drug retention, the rigidity of the liposome bilayer membrane can be increased by using lipids containing longer, more saturated fatty-acid chains with a higher T_c. Cholesterol can either decrease or increase the permeability of a lipid membrane, depending on the T_c of the lipid and also the environmental temperature. When lipid bilayers are in the liquid-crystalline or fluid state, permeability is decreased by cholesterol as the fatty-acid chains undergo tighter packing (Chapman and Penkett, 1966). Membranes in their gel state, i.e., below T_c, are fluidized by cholesterol, which increases the distance between the lipid polar head groups, thereby allowing greater mobility of the fatty-acid chains. The results of the present study are in agreement with the expected effects of cholesterol. The incorporation of cholesterol was found to destabilize, in terms of enhanced GSH leakage, liposomes composed of DSPC and DPPC, which were in a gel state at 37°C.

The relatively 'low entrapment' of GSH we observed using DMPC liposomes has also been noted for other solutes such as sodium cromoglycate (Taylor et al. 1990b) and metaproterenol sulfate and sucrose (Abra et al., 1990). This apparent low entrapment is very likely due to the particularly high permeability of DMPC liposomes at 23°C (T_c) which is the same as or close to room temperature where the entrapment efficiency is usually performed (Blok et al., 1976). The incorporation of cholesterol into DMPC liposomes, at levels above 20 mol%, however, is known to result in a very significant broadening of the lipid's phase transition (Mabrey et al., 1978) and we have also demonstrated that the incorporation of 25 mol% cholesterol in DMPC liposomes practically abolishes the sharp transition at 23°C (Yung et al., 1985). Therefore, the abolishment of T_c with the incorporation of 30 mol% cholesterol may well account for the much improved entrapment of GSH observed in this study.

Cholesterol reduced the vesicle diameter of DSPC and DPPC liposomes. A condensing effect of cholesterol has been described for saturated and mono-unsaturated phospholipids (Yeagle, 1985). However, a study of extruded PC liposomes demonstrated that cholesterol increased the vesicle size (Morii et al., 1983). Thus, it seems that cholesterol may increase the size of extruded MLV vesicles composed of phospholipids in the gel state, such as DMPC and PC, but will decrease the size of vesicles composed of phospholipids in the liquid-crystalline state, e.g., DSPC and DPPC.

The selection of lipids for preparing liposomes to be used as a drug delivery system depends on many factors, including the availability, cost, safety, and ease of utilization of the lipids. Entrapment efficiency is, of course, also an impor-

tant consideration. From the present investigation, the relatively poor entrapment of GSH in DMPC liposomes suggests that DSPC or DPPC would be a better choice for encapsulating a relatively small, water-soluble compound, under conditions of storage at or below room temperature. DMPC liposomes are more susceptible than DSPC and DPPC liposomes to leakage of GSH when the T_c was traversed, as the temperature declined from 33°C (10°C above T_c) during liposome preparation to 5°C for the determination of entrapment efficiency and storage. Since the T_c of DMPC is 23°C, liposomes composed of this phospholipid would be highly susceptible to leakage at room temperature rendering the use of DMPC liposomes impractical as a drug carrier.

With regard to leakage kinetics, it would seem that for most applications, release from DSPC vesicles may be too slow for adequate drug release. Thus, of the lipid compositions considered in these experiments, DPPC appears to have the greatest potential for effective drug release. The fact that DPPC is a natural component of lung surfactant suggests that it may be particularly suitable for pulmonary delivery systems, although, for reasons mentioned above, its interaction with cellular components needs to be assessed. A mixture of DPPC and DSPC liposomes, the former mediating an immediate release and the latter mediating a slow, gradual release, is also a possible approach. By electing to include or exclude cholesterol, and by selecting the amount of cholesterol for inclusion, it may be possible to tailor the liposomes to manifest release kinetics for optimal biological effect.

From our investigation, we find it interesting that there is a non-cellular component in BAL which destabilizes phospholipid vesicles. Since the fluid component is likely the first point of contact for liposomes introduced into the alveolar spaces, these observations may reflect at least the initial release kinetics of entrapped solutes. It remains to be established whether in vitro incubations of liposomal preparations with BAL can serve to indicate or predict in vivo release in the lung. Animal studies are necessary to determine the efficacy of this in vitro system as a means of obtaining preliminary information on the release characteristics of a liposomal preparation intended for pulmonary delivery.

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