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Distribution studies of liposome-encapsulated glutathione administered to the lung

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Summary

To test the feasibility of using liposomes for the pulmonary delivery of glutathione, we compared the distribution of free and liposome-encapsulated glutathione after intratracheal instillation of the ^{3}H-labelled tripeptide to rats. Free glutathione rapidly left the lung and entered the circulation. Liposomally delivered glutathione remained in the lung for a prolonged period, most likely by remaining in encapsulated form, and entered the bloodstream only after release from the liposomes. Excretion of ^{3}H into the urine after liposomally delivered glutathione was associated with free glutathione or its degradation products. Glutathione from liposomes instilled into the trachea was not distributed evenly among different lobes of the lung; however, the variation was not greater than 4-fold. These results demonstrate that exogenously supplied glutathione can be confined to the lung by means of a liposomal delivery system.

Introduction

Liposomes are microscopic, artificially created vesicles, composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Several methods of preparing liposomes have been developed. By varying the method of preparation and the lipid composition, vesicles of different sizes, encapsulation efficiencies and biological activities can be obtained (Szoka and Papahadjopoulos, 1980). As a drug delivery system, liposomes can significantly change the pharmacokinetic and pharmacodynamic fate of a compound (Juliano and Stamp, 1978). Liposomes have been used to target drugs to specific organs, delay loss of rapidly cleared drugs, enhance absorption of topically applied drugs, reduce toxicity, and potentiate therapeutic potency or immunogenicity (Shek and Barber, 1986; Emmen and Storm, 1987).

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Targeted delivery to the pulmonary system is desirable for several reasons. The lung is extremely susceptible to toxic insults from the environment. Toxic damage and disease which are localized within the lungs would benefit from organ-specific delivery of therapeutic or protective agents. For example, antineoplastic agents used for treating lung tumors are associated with significant extrapulmonary toxicity (Cooper et al., 1986); selective delivery to the lung may improve therapeutic efficacy and reduce the incidence and severity of side-effects.

Enhanced drug uptake by the lung has been achieved by the intravenous injection of drug-containing liposomes, larger than 1 μm in diameter (Hunt et al., 1979; Abra et al., 1984) or by incorporating certain sugar derivatives into the lipid bilayer (Mauk et al., 1980; Takada et al., 1984). Intratracheal instillation (Juliano and McCullough, 1980; Padmanbhan et al., 1985) and aerosolization of liposomes (Debs et al., 1987; Wyde et al., 1988) have also been used to achieve selective delivery to the lungs. Following instillation into the respiratory system, liposome-encapsulated cytosine arabinoside has been shown to be retained in the lung significantly longer than the free drug (Juliano and McCullough, 1980). Liposomal entrapment of superoxide dismutase has been shown to enhance the protective effect of the enzyme against hyperoxic lung injury, by prolonging its half-life (Turrens et al., 1984) and, presumably, by providing a transmembrane vector for intracellular delivery (Freeman et al., 1983).

Intracellular glutathione is of major importance in protecting cells against injury by toxic chemicals, radiation, reactive oxygen species and free radicals (Sies and Wendel, 1978). Attempts to utilize the protective or therapeutic potential of the tripeptide have been hindered by its short circulating half-life (Wendel and Cikryt, 1980) and its inability to cross cell membranes (Puri and Meister, 1983). Exogenously supplied glutathione is hydrolyzed rapidly in the kidneys into its constituent amino acids which are then redistributed and resynthesized to the tripeptide in the liver (Hahn et al., 1978; Griffith and Meister, 1979). Thus, in order for glutathione to retain its efficacy at a specific target site, the rapid hydrolysis must be prevented or at least delayed. Liposome encapsulation may provide a means of achieving this and, indeed, has been shown to significantly improve the hepatoprotective effect of intravenously administered glutathione against acetaminophen-induced liver toxicity (Wendel and Jaeschke, 1982).

Since pulmonary glutathione serves as an endogenous protective agent against lung injury (Boyd et al., 1982), its augmentation by exogenously administered glutathione may provide additional protection. In the present report, we describe findings comparing the distribution of free and liposome-entrapped glutathione following their administration to rats by intratracheal instillation. In particular, we investigated the effect of liposomal encapsulation on the retention of glutathione within the lungs.

Materials and Methods

Male Wistar rats weighing 225–250 g were purchased from Charles River Canada (St. Constant, Que.). The animals had free accesss to laboratory chow and drinking water. They were acclimatized to their cages for 1 week before starting the experiment. 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* as prepared by the Canadian Council on Animal Care.

Egg yolk phosphatidylcholine, cholesterol, stearylamine, glutathione and catalase were obtained from Sigma (St. Louis, MO). [^{3}H]Glutathione (reduced form, 0.9–1.1 Ci/mmol), [^{14}C]-cholesterol (0.06 Ci/mmol) and liquid scintillation supplies were from New England Nuclear (Boston, MA). All reagents and solvents were of analytical grade.

Liposome preparation

Liposomes were prepared from a mixture of phosphatidylcholine, cholesterol and stearylamine in a 6 : 3 : 1 molar ratio. This lipid composition was chosen because a relatively similar formulation has been shown to be effective for drug

delivery to the lung by intratracheal instillation (Juliano and McCullough, 1980). The lipids were dissolved in chloroform-methanol (2:1, v/v) and ^{14}C-labelled cholesterol (0.7–1.3 μCi) was added as a tracer. The lipid mixture was dried under helium in a 40°C water bath. The vessel was periodically rotated manually to obtain a film of lipid adhering to the inside of the vessel. The vessel was then placed under vacuum for 90 min to remove trace solvent. Multilamellar vesicles were prepared by vortexing the dry lipid with a freshly prepared 0.4 M solution of reduced glutathione in 5 mM potassium phosphate buffer of pH 6.5 containing 30 mM EDTA and a trace amount of ^{3}H-labelled reduced glutathione (25 μCi). The multilamellar vesicles were frozen in liquid nitrogen and thawed in a 40°C water bath. The freeze-thaw cycle was repeated four times. The freeze-thaw cycle gave rise to multilamellar vesicles with a larger aqueous compartment and therefore greater entrapment efficiency (Hope et al., 1985; Mayer et al., 1986). The resulting vesicle preparation was transferred to an Extruder (Lipex Biomolecules, Vancouver, BC) connected to a helium cylinder, and extruded through two stacked polycarbonate filters of 400 nm pore size. The preparation was passed through the filters 10 times, using a helium pressure of 100–500 lb/in^2. The extrusion process favors a more homogeneous size distribution. The vesicle size distribution was determined by quasi-elastic light scattering using a Coulter N4SD particle size analyzer. A unimodal distribution was observed with a mean vesicle diameter of 195 ± 62 nm (SD, $n = 3$). Unentrapped glutathione was removed by washing the liposomes twice in 5 mM potassium phosphate buffer (pH 6.5) and pelleting at $110\,000 \times g$ for 1 h at 5°C in a Beckman L8-70 Ultracentrifuge. The vesicle size distribution was not affected by ultracentrifugation. Aliquots of supernatants and pellets were counted for ^{3}H and ^{14}C employing a Beckman LS-5801 liquid scintillation spectrometer. The glutathione entrapment efficiency was 0.67 ± 0.13 (SD, $n = 5$) μmol/μmol lipid. In several experiments, glutathione entrapment was also measured using the Ellman assay for sulfhydryl groups (Ellman et al., 1961). The results were entirely consistent with those obtained using the radiolabel as a marker, indicating no appreciable loss of sulfhydryl groups during preparation. The liposomes were diluted with a 5 mM potassium phosphate buffer to give a final glutathione concentration of 65 mM. The lipid concentration of the diluted liposomes was 95–115 μmol/ml.

Treatment of animals

Rats were anesthetized with 50 mg/kg i.p. of sodium pentobarbital. A 2 cm incision was made to expose the trachea which was cannulated with a 9 cm length of PE-200 polyethylene tubing (i.d. = 1.4 mm, o.d. = 1.9 mm). The tip of the cannula was positioned approximately at the tracheal bifurcation. A 9.2 cm length of PE-50 polyethylene tubing (i.d. = 0.58 mm, o.d. = 0.97 mm), attached at one end to a 250 μl glass Hamilton syringe, was passed down the tracheal cannula. Each animal then received, via intratracheal instillation, 0.1 ml of either free glutathione solution (65 mM) or liposome-encapsulated glutathione solution followed by 0.05 ml of 0.85% saline to rinse the syringe and tubing. The instillation was carried out slowly, over 1–3 min.

For rats designated for killing at 0.5, 1, 3, and 6 h, the cannula was tied in position, the incision closed with surgical staples and the excess cannula cut to leave an approx. 1 cm protrusion. For rats to be killed at later intervals (24 and 48 h), the cannula was removed and the tracheal incision closed with one or two sutures. Animals were placed into metabolic cages for urine collection.

Prior to killing, rats were anesthetized with i.p. sodium pentobarbital. The thorax was opened and approx. 2 ml of blood was collected into a heparinized test tube by cardiac puncture. The lungs and trachea were excised and urine in the bladder was collected by syringe. All biological samples were temporarily stored at −20°C.

Preparation of biological samples

Wet weights of the trachea and individual lobes of the lungs were recorded. In the rat, the left lung consists of a single large lobe, while the right lung is made up of four separate lobes, namely the upper lobe (UL), the middle lobe (ML), the lower lobe (LL), and the cardiac lobe (CL). The tissues

were minced and transferred into 22-ml glass counting vials to which 0.1 ml Protosol tissue solubilizer was added. The vials were incubated in a shaking water bath at 45–55°C for 4–5 h or until the tissue had dissolved. After cooling, 15 ml of Biofluor scintillation cocktail were added, followed by 0.5 ml of 1 N HCl. Blood samples were prepared by solubilizing a 0.2 ml aliquot of whole blood in 0.5 ml of a 1 : 2 (v/v) Protosol : ethanol mixture for 1 h at 45–55°C. To decolorize blood samples (and also samples from the large single lobe of the left lung and LL of the right lung, which were larger than the other lobes and formed solutions which were too intensely colored for liquid scintillation counting), 0.2 ml of 30% H_2O_2 was added dropwise to vials after solubilization was completed. The vials were loosely capped and incubated for another 30 min. After cooling, 15 ml of Biofluor and 0.5 ml of 1 N HCl were added. If necessary, vials were allowed to stand for 1–3 days before counting, to allow chemiluminescence to decrease. In some cases, 7500 units of catalase were added to vials after treatment with H_2O_2. Catalase increased the rate of decay of chemiluminescence caused by peroxides (Grower and Bransome, 1970). The total recovery of radioactivity from the blood was calculated (assuming a blood volume of 15 ml for rats of approx. 225 g) based upon the reported blood volume for the rat of 6.7 ml per 100 g body weight (Creskoff et al., 1949). Aliquots of urine were counted in Aquasol. The total volume of urine collected was used to calculate the recovery of radioactivity.

Liquid scintillation counting

The samples were counted in a Beckman LS-5801 liquid scintillation spectrometer with stored quench curves to calculate automatically dpm from cpm. Both ^{14}C and ^{3}H were counted with a 2 σ value, indicating that the 95% confidence level for the count was $\pm 2\%$. The instrument is equipped with a random coincidence monitor (RCM) which indicates the percentage of cpm attributable to non-radioactive light-producing events. For these experiments, a maximum RCM value of 10% was accepted. If the value was higher, the sample was recounted at a later date, usually 1–3 days, when the chemiluminescence had decayed sufficiently.

Results

Distribution of radioactivity after administration of free and liposome-encapsulated [^{3}H]glutathione

Liposome encapsulation of [^{3}H]glutathione altered its retention in the lung and also affected its distribution to the blood and urine (Fig. 1). Free [^{3}H]glutathione, administered to the respiratory system, rapidly disappeared from the lung, leaving less than 10% of the initial ^{3}H dose remaining at 1

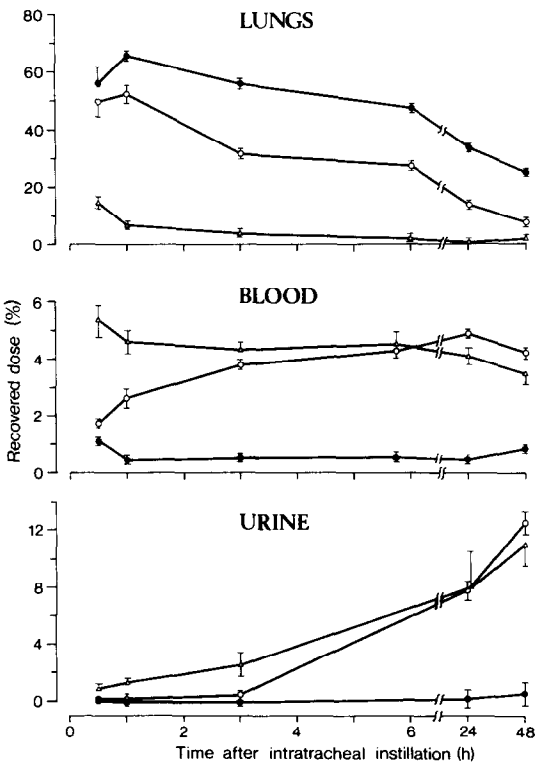

Fig. 1. Recovery of radioactivity from the lung, blood and urine of rats instilled intratracheally with ^{14}C-labelled liposomes containing entrapped [^{3}H]glutathione (GSH), or with free [^{3}H]GSH. Each animal received a dose of 2 mg of GSH in a volume of 100 μl. Each point represents the mean percentage of recovered dose ± SE. ^{14}C (●———●) and ^{3}H (○———○) recovered from animals instilled with ^{14}C-liposomes containing entrapped [^{3}H]GSH, and also ^{3}H (△———△) recovered from animals instilled with free [^{3}H]GSH. For the determination of lung and blood levels of radioactivity, three to nine animals were killed at 0.5, 1, 3 and 6 h after administration, and two to five animals at 24 and 48 h. For the determination of urine levels of radioactivity, two to six animals were killed at each of the various time points. Urine values represent accumulated radioactivity from time zero.

h. In contrast, after the administration of liposomal [^{3}H]glutathione, approx. 50% of the initial tritiated dose remained in the lung at 0.5 and 1 h. At 24 h, 14% of the dose was still present and at 48 h, 8%. After administration of free [^{3}H]glutathione, radioactivity had dropped to near-background levels by 24 h.

Comparison of ^{3}H and ^{14}C recoveries after the administration of liposomal glutathione demonstrated that the two isotopes were eliminated from the lung in parallel (Fig. 1, upper panel). At 0.5 and 1 h, the recovery of ^{14}C was 7–8% higher than that of ^{3}H. At later times, the recovery of ^{14}C was 17–20% higher as compared to ^{3}H. This suggests that the majority of [^{3}H]glutathione, still present in the lung, was associated with liposomes. A small proportion may have undergone early, rapid release from the liposomal vesicles.

In the blood, approx. 5% of the dose of ^{3}H radioactivity after administering free [^{3}H]glutathione was detected at 0.5 h (Fig. 1, middle panel). The level declined only slightly over 48 h. This constant level may reflect a continuous redistribution as glutathione uptake, utilization and release occurred in various tissues. Thus, it should be noted that the ^{3}H label in the blood may not represent intact glutathione. The appearance of tritiated counts in the blood was slower after the intratracheal administration of liposomal glutathione. Less than 2% of the dose was present in the blood at 0.5 h and this increased to a maximum of 5% at 24 h. Although the proportion of the dose recovered from the blood was low, there was a significant difference between the free- and liposomal glutathione-treated animals at 0.5 h ($p < 0.001$) and 1 h ($p < 0.005$). The initial higher levels of ^{3}H in the blood following the administration of glutathione in the free form indicate that elimination from the lung is faster in comparison to the liposomal form.

In contrast to the lung observations, the blood data did not show a parallel elimination of ^{3}H and ^{14}C after the administration of liposomal glutathione. The ^{14}C-lipid level was at a constant low value of about 0.5% of the administered dose throughout the 48 h period. Although the total increase in ^{3}H levels during this period was small, the pattern of ^{3}H appearance in the blood had no correlation with the pattern of ^{14}C appearance. Therefore, any glutathione in the blood was not liposome-associated.

In the urine, after administration of liposomal [^{3}H]glutathione, only trace amounts of ^{3}H appeared during the first 3 h (Fig. 1, lower panel). After free [^{3}H]glutathione, the recovery of ^{3}H from the urine was initially slightly higher, reflecting the more rapid loss of free glutathione from the lung. However, at 24 and 48 h, urinary recovery of ^{3}H was similar for both preparations, and only trace amounts of ^{14}C were present after liposomal glutathione. This suggested that glutathione administered in liposome-encapsulated form was excreted in the urine free of liposomes, either as glutathione or as its degradation products.

The data for ^{3}H recoveries from the lung are plotted on a semi-logarithmic scale in Fig. 2. The loss of ^{3}H from the lung after both free glutathione and liposomal glutathione was a bi- or multi-phasic process. After free [^{3}H]glutathione administration, almost all of the radioactivity was removed from the lung very rapidly, within 6 h. After liposomal [^{3}H]glutathione, approx. two-thirds of the administered radioactivity was eliminated within 3 h. The remaining one-third of

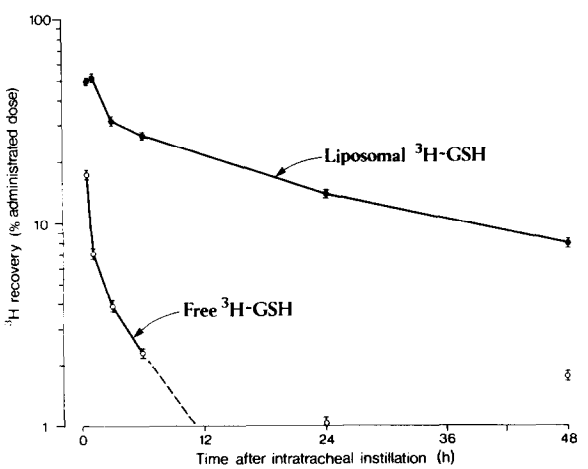

Fig. 2. Clearance of ^{3}H from the lung after administration of liposome-encapsulated or free [^{3}H]GSH. Each rat received an intratracheal instillation of 2 mg of GSH in a volume of 100 μl. Data are plotted on a semilogarithmic scale. Each point represents the mean percentage of recovered dose ± SE. At 0.5, 1, 3 and 6 h, four to nine animals were killed. At 24 and 48 h points, two to five animals were killed.

the dose was cleared very gradually, with an estimated half-life of 27 h.

Distribution of ^3H to individual lobes of the lung after liposomal [^3H]glutathione

To follow the distribution of [^{3}H]glutathione in different lobes of the lung, the ^{3}H radioactivity was counted for each individual lobe. Fig. 3 illustrates the ratio of the fraction of ^{3}H recovery within each lobe divided by the fractional mass of that lobe. The pattern of distribution did not appear to vary with time. Therefore, the average of ratios for 20 rats killed at various times is presented. This average ratio varied 4-fold between the various lobes. Although the inter-animal variation was large, it appears that the right upper lobe received a disproportionately greater amount of ^{3}H, and the right middle and cardiac lobes, disproportionately lesser amounts. These discrepancies in the distribution of liposomally delivered [^{3}H]glutathione could be related to the anatomy of the bronchioles and their gravitational position during intratracheal instillation.

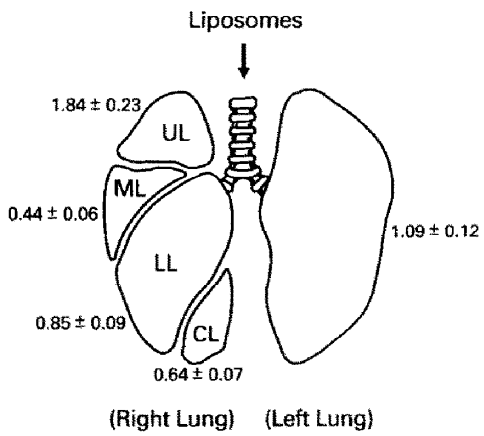

Fig. 3. Distribution of ^{3}H in different lobes of the rat lung after administration of liposome-encapsulated [^{3}H]GSH. Each animal was instilled intratracheally with 100 μl of liposomes as described in Fig. 1. In the rat, the left lung consists of a single large lobe, and the right lung is made up of four lobes, namely, the upper lobe (UL), middle lobe (ML), lower lobe (LL), and cardiac lobe (CL). In the schematic diagram, each number outside a lobe is the ratio obtained by dividing the ^{3}H recovery from that lobe (expressed as a percentage of total ^{3}H recovery for the lung) with the lobe mass (expressed as a percentage of the total lung mass). The results show the mean ± SE for rats ($n = 20$) killed at various times from 0.5 to 24 h. The 48 h data were not included since ^{3}H recoveries at this time were low.

Discussion

This study demonstrated the use of liposomes to achieve organ-selective delivery of glutathione. After intratracheal instillation, free glutathione rapidly left the lung, whereas the encapsulated tripeptide remained in the lung for a prolonged period. These results are in agreement with a previous study of liposome-encapsulated [^{3}H]cytosine arabinoside (Juliano and McCullough, 1980).

The biphasic pattern of disappearance of administered liposomal compound from the lung observed in this investigation is also in agreement with an earlier study (Juliano and McCullough, 1980). However, the reason for the biphasic pattern is unknown. It has been suggested that the interaction of proteins with the liposome surface may produce a two-stage pattern of solute release (Hunt, 1982). The first stage involves an initial rapid adsorption of protein to the liposome surface, creating a localized change in surface tension which causes a transient high permeability. This is then followed by a rearrangement of the adsorbed protein and membrane lipid to a final stable configuration which is accompanied by a slower sustained release.

Our recovery of the administered liposomes at 0.5 h was only about 60% and that of the liposomally administered glutathione, 50%. Recovery from the trachea of ^{14}C radioactivity associated with lipid was less than 1%. Washes of the syringe used for administering the liposomes and washes of the tracheal cannula recovered an additional 10% of the dose, maximally. Therefore, we are confident that nearly 90% of the calculated dose of liposomes reached the lung. Thus, there was an early rapid removal of liposomally administered glutathione from the lung. This may be partially attributed to leakage from the liposomes, subsequent uptake into the circulation and redistribution. There was also an early rapid loss of the actual lipid vesicles.

The removal of intact liposomes from the lung may occur by mucociliary clearance or phagocytosis of vesicles by pulmonary macrophages (Miyamoto et al., 1988). Uptake of liposomal phospholipids by granular pneumocytes (Type II cells) and recycling of the phospholipids for surfactant

synthesis (Chander et al., 1983) may account for the higher recovery of lipid compared to glutathione at the various times examined. Loss of liposomally administered glutathione might be mediated by any of the above processes as well as by leakage of glutathione across a lipid bilayer which is destabilized in the pulmonary milieu. Liposome breakdown would also have released glutathione locally in the lung. If mucociliary clearance of intact liposomes occurred to any significant degree, this would be of little benefit for elevating glutathione levels in the lung. On the other hand, phagocytosis of glutathione-containing liposomes by alveolar macrophages may be desirable for protecting the lung from oxidant-induced injury. Alveolar macrophages are susceptible to damage from reactive oxygen species and upon injury, release factors which lead to further tissue damage (Harada et al., 1983). The relative contribution of various processes to the disappearance of the liposomally administered glutathione remains to be established.

Liposomes instilled into the trachea at or near the tracheal bifurcation, in a volume of 0.15 ml, did not distribute uniformly to the different lobes of the lung. Non-uniform distributions in the rat lung after intratracheal instillation were also reported by McCullough and Juliano (1979) for a suspension of liposomes and by Brain et al. (1976) for a suspension of colloid particles. In our method of administration, liposomes were suspended in a carrier fluid. Gravity can influence the deposition of the liposomes since it determines the movement of fluid into the lung. The carrier liquid is quickly absorbed into the pulmonary circulation while the liposomes remain on the internal surfaces of the lung (Brain et al., 1976). In anesthetized animals placed head-up on a board slanted at 20° from the vertical, intratracheal instillations produced greater deposition of particles in the top portions of the lung than in the lower regions (Brain et al., 1976). In our experiments, the anesthetized rats were placed on their backs. This produced a more uniform interlobe distribution than that of the study by Brain and co-workers in which the animals were tilted. Pulmonary distribution also appears to depend on the volume of fluid instilled: the larger the volume of fluid, the deeper the penetration to the peripheral areas of the lung (Baxter and Port, 1974). On the other hand large volumes of fluid will produce greater respiratory distress and cell injury. We chose an instillation volume that caused neither mortality in any animal nor any gross pathological damage in the lung. However, we did not examine the actual distribution of liposomes within each lobe.

Aerosol delivery has been shown to provide more uniform pulmonary distribution of particles than intratracheal instillation (Brain et al., 1976). After intratracheal delivery to anesthetized rats of aerosolized ^{14}C-labelled liposomes, we observed less than 2-fold variation in the distribution ratio for the different lobes of the lung (unpublished observations). Aerosolized liposomes have been used to deliver drugs to the respiratory system (Debs et al., 1987; Wyde et al., 1988). However, there are disadvantages to aerosol delivery which include difficulty in controlling the dose in experimental animals and restriction to delivery of relatively small doses. The potential for significant deposition in the bronchi and bronchioles (Wyde et al., 1988) may be a disadvantage if the target tissue is at the alveolar level. For pulmonary delivery to rodents, the technique of intratracheal instillation allows precise control of dose and the introduction of large doses in a short time. Of course, intratracheal instillation can have only specialized applications in human use and aerosol delivery is essential for any wider therapeutic application.

Liposome-encapsulated glutathione may be useful as a therapeutic agent for protecting the lung against certain types of chemical and oxidative toxicity. Other studies have demonstrated that liposome entrapment of superoxide dismutase and catalase protected animals against pulmonary toxicity induced by hyperoxia (Turrens et al., 1984; Padmanbhan et al., 1985). Delivery of liposome-encapsulated glutathione to the lung might likewise augment the cellular antioxidant defense mechanisms and provide enhanced protection against free radical-mediated oxidative injury. The potential efficacy of this delivery system depends on maintaining the integrity of the functional thiol group of glutathione in lung tissue. If a protective effect can be demonstrated, the assessment of lung

levels of glutathione may suggest the mechanism of protection. In vitro, exogenous glutathione provided rat alveolar Type II cells with protection against damage by paraquat (Hagen et al., 1986), a compound which mediates its lung-selective toxicity by generating reactive oxygen species. Based on the results of the present study, it might be worthwhile to assess the in vivo efficacy of liposome-encapsulated glutathione in protecting the lung against paraquat-induced injury.

Overall, the results of this investigation suggest that liposomes may be of potential benefit for pulmonary delivery by prolonging retention of the encapsulated agent in the lung. It is conceivable that liposome encapsulation may enhance the therapeutic efficacy of pulmonary drugs by producing a local pharmacologic response within the target organ and minimizing extrapulmonary adverse effects.

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