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The deposition and clearance of liposome entrapped ^{99m}Tc-DTPA in the human respiratory tract

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Summary

The size characteristics of aerosols produced by nebulising liposomes from five jet nebulisers have been investigated. The size and size distribution of the aerosols was determined by the design of the nebuliser and the gas flow rate employed to drive the nebuliser, rather than on the properties of the liposome preparations themselves. The clearance of inhaled dipalmitoylphosphatidylcholine/cholesterol liposomes containing the aqueous phase marker ^{99m}Tc-DTPA was studied by gamma-scintigraphy following inhalation by three healthy volunteers. Approx. 45% of originally deposited radioactivity remained in the lungs after 24 h. This represents the fraction of the radiolabel remaining in intact alveolar-deposited vesicles, since free ^{99m}Tc-DTPA was removed from the airways with a half-life of 75 min.

Introduction

Liposomes seem a particularly appropriate carrier system for pulmonary drug delivery, as they can be prepared from phospholipids present endogenously in the respiratory tract as components of pulmonary surfactant. Studies of the toxicological effects of inhaled liposomes after acute administration to man, produced no observable changes in pulmonary function up to 6 h post-inhalation (Thomas et al., 1991). After chronic inhalation of liposomes by animals, no adverse effects in terms of survival, histopathology or macrophage function were detected (Myers et al., 1993).

Studies of pulmonary deposited liposomes have indicated that liposome encapsulation of drug prior to administration can produce modulated pulmonary absorption, resulting in localised drug action in the respiratory tract (Juliano and McCullough, 1980), a prolonged presence in the systemic circulation (Meisner et al., 1989) and decreased incidence of systemic side effects (McCaldon et al., 1989).

For a material to penetrate to the bronchioles and alveoli, particles must be of a 'respirable' size, usually 5–6 μm or less (Stahlhofen et al.,

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1980). Metered dose inhaler systems based on phospholipid/chlorofluorocarbon solutions or dehydrated liposomes suspended in chlorofluorocarbons have been developed as a means of liposome delivery to the human respiratory tract (Farr et al., 1985; Radhakrishnan et al., 1990). Dehydrated liposomes may also be delivered from dry powder inhalation devices (Radhakrishnan et al., 1990). However, nebulisation of liposome suspensions to produce aerosols is the simplest method for such delivery and has been used in previous studies of liposome delivery to the human respiratory tract (Farr et al., 1985; Gilbert et al., 1988; Taylor et al., 1989).

Farr et al. (1985) in a gamma scintigraphy study, nebulised liposomes, having their external phospholipid bilayer labelled with ^{99m}Tc, to healthy volunteers. The deposition and clearance of both dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs) were similar, being dependent on the site of deposition within the airways, which was determined by the particle size of the aerosol, rather than the size of vesicles. Radioactivity remaining in the lung after 20 h was assumed to represent alveolar deposited vesicles. The measured values for alveolar retention were less than predicted by extrapolation of short term clearance profiles, which may indicate removal of vesicles from the alveolar region or a time-dependent dissociation of the surface-associated radiolabel.

In a study of a DPPC/cholesterol formulation of sodium cromoglycate, inhaled from a Hudson jet nebuliser by healthy volunteers, drug was detectable in plasma up to 25 h post-inhalation (Taylor et al., 1989). An equivalent dose of drug inhaled as a solution could not be detected after 8 h, indicating that liposome entrapment of sodium cromoglycate prior to pulmonary administration prolonged drug retention within the lungs and altered its pharmacokinetics.

In this study a double-headed Rota gamma camera was employed to investigate the fate of inhaled liposomes, containing the aqueous phase marker ^{99m}Tc-DTPA. Using a water-soluble radiolabel allows the integrity of the pulmonary deposited vesicles to be assessed.

Materials and Methods

Materials

Unless otherwise stated materials in this study were AnalaR grade and obtained from BDH (U.K.). Egg phosphatidylcholine (EPC; about 90%, Sigma Chemical Co. Ltd, U.K.) was purified as described by Bangham et al. (1974). Dipalmitoyl-L-α-phosphatidylcholine (DPPC; 99 + %) and cholesterol (Chol; 99 + %) were obtained from Sigma Chemical Co. Ltd (U.K.). Solutions of ^{99m}Tc as pertechnetate were eluted from a generator (Elumatic III, Compagnie ORIS Industrie, France) with sterile 0.9% w/v sodium chloride solution (Antigen Europe, U.K.). This was added to sterile diethylenetriamine pentaacetic acid (DTPA; Amersham International, U.K.), shaken and allowed to stand for 15 min to produce ^{99m}Tc-DTPA.

All water was deionised (Whatman WR50 RO/Deioniser, Whatman, U.K.).

Preparation of liposomes

All liposome preparations had a total lipid concentration of 40 mg ml^{-1}.

The required amount of phospholipid, with cholesterol in equimolar quantities if required, was weighed into a quickfit round bottomed flask and dissolved in a small volume of chloroform. Organic solvent was removed at reduced pressure, on a rotary evaporator at 40°C, to form a thin lipid film. The aqueous phase (deionised water or ^{99m}Tc-DTPA in sterile 0.9% w/v sodium chloride solution) was added at 40°C for EPC or 50°C for DPPC films. The flask was maintained at that temperature for 30 min, sonicated in an ultrasonic bath for 2 min, then allowed to stand for a further 30 min at the same temperature. This method resulted in the production of MLVs.

The particle size of some EPC formulations and all ^{99m}Tc-DTPA containing DPPC/Chol liposomes was reduced by extrusion through polycarbonate membrane filters (Nucleopore Inc., U.S.A.) held in a 25 mm ultrafiltration cell (Amicon Corp., U.S.A.), until the diameter of the vesicles approximated the pore size of the filter (Szoka et al., 1980).

For the in vivo study liposomes were passed through a 3 μm pore filter five times and a 1 μm pore filter 10 times. The vesicle size of liposomes was determined by laser diffraction analysis (Malvern 2600c, Malvern Instruments, U.K.).

Unentrapped ^{99m}Tc-DTPA was removed from liposome preparations by ultrafiltration for 18 h (Diaflo PM10 membrane, Amicon Corp., U.S.A.), held in a 10 ml cell (Amicon Corp., U.S.A.). The liposomes were reconstituted to 6 ml with sterile 0.9% w/v sodium chloride solution.

Characterisation of liposomal aerosols produced by jet nebulisers

The following nebulisers were used to generate aerosols from deionised water or EPC MLV formulations: Hudson (Henleys Medical Supplies Ltd, U.K.); Respirgard II (Marquest, U.S.A.); System 22 with or without Optimist filter (Medic-aid, U.K.) and Turret (Medic-aid, U.K.).

Aerosols were produced from 6 ml (Respirgard II) or 8 ml (all other nebulisers) of liquid using compressed nitrogen at various gas flow rates. Aerosol size was measured with a laser diffraction particle size analyzer (Malvern 2600c, Malvern instruments, U.K.).

Assessment of in vitro release of ^{99m}Tc-DTPA

The extent of leakage of entrapped ^{99m}Tc-DTPA from vesicles was measured by diluting the 6 ml of liposome preparation to 50 ml with 0.9% w/v sodium chloride solution and placing in an incubator at 37°C for 24 h. 10-ml samples were taken and placed in a well counter to determine total radioactivity. The unentrapped radioactivity was separated from the liposomes by ultrafiltration (Diaflo PM10 membrane, Amicon Corp., U.S.A.). The amount of isotope released over 24 h was calculated, allowing for radioactive decay.

Characterisation of aerosols produced by Respirgard II nebulisers from radiolabelled liposomes

Aerosols were generated from 6 ml of ^{99m}Tc-DTPA containing liposome preparation, immediately after reconstitution, using a Respirgard II nebuliser fitted with a mouthpiece. The nebuliser was driven by nitrogen from a compressed gas cylinder at a flow rate of 6 l min^{-1} for 20 min and the aerosol directed into a calibrated multi-stage liquid impinger (MLI). Air was drawn through the impinger at 60 l min^{-1}, via a terminal filter, by means of a vacuum pump. The radioactivity deposited on each stage of the impinger was measured using a well counter, and the mass median aerodynamic diameter (MMAD) and geometric standard deviation (σ_g) of the aerosol calculated.

In vivo study

The volunteer study received ethics committee approval and volunteers participated with written informed consent. Imaging of the lungs was performed using a Siemens Rota gamma camera system with two opposed ZLC 37-tube detectors. Both detectors were fitted with low energy parallel hole collimators. Data processing was performed with an on-line ADAC digital computer (DPS 3300).

Three healthy non-smoking males aged between 18 and 30 years took part in the study. Each inhaled an extruded DPPC/Chol (1:1) liposomal formulation containing ^{99m}Tc-DTPA, from a Respirgard II nebuliser driven by compressed air at 6 l min^{-1} for 18 min, to ensure lung deposition of 3–4 MBq of ^{99m}Tc. During nebulisation, and for 42 min post-nebulisation, the volunteer remained seated between the two heads of the gamma camera. 60 1-min frames were taken during this time period. 60 single photon emission tomographic (SPET) images were then acquired with the volunteer lying down and the camera heads rotated. Images were taken at 6° intervals with one head only. 5-min frames were taken, using both heads, every 0.5 h commencing 4 h post-nebulisation and continued until 8 h post-nebulisation. 15-min frames were taken at 23 and 24 h post-nebulisation.

One volunteer inhaled on a separate occasion 3–4 MBq of free ^{99m}Tc-DTPA delivered by a Respirgard II nebuliser. 60 1-min frames were taken, commencing with the onset of inhalation. 2-min images were then acquired every 5 min between 1 and 2 h post-nebulisation. Subsequently, 2-min frames were taken every 20 min, 2–5 h post-nebulisation.

In each experiment, the results were analysed in terms of the quantity of radioactivity remaining in each lung. Camera images were computed to produce a visual image. Anterior and posterior measurements were taken and used to calculate the geometric mean count. Regions of interest were defined around the left and right lung and the geometric mean activity within these regions quantified. All data were corrected for background activity and radioactive decay.

Results and Discussion

Aerosol analysis

With jet nebulisers, the factors determining the emitted aerosol size are the design of nebuliser (Clay et al., 1983b) and rate of gas flow through the nebuliser (Clay et al., 1983a). Properties of the liquid being nebulised, such as surface tension and viscosity, may also influence the size characteristics of the aerosol (Davis, 1978; Newman et al., 1987).

Except for the Turret nebuliser at the highest gas flow rate, the Hudson, System 22 and Turret nebulisers produced aerosol droplets from deionised water between 2.4 and 4.2 μm (Table 1). Aerosols in this size range have a high probability of deposition in the respiratory and terminal bronchioles and alveoli (Stahlhofen et al., 1980). The Respirgard II and System 22 with Optimist nebulisers have been developed for delivery of drugs used in the treatment of alveolar infections, and consequently produced aerosols with MMDs of 2 μm or less.

TABLE 1

Size characteristics of aerosols produced by atomization of deionised water in jet nebulisers

Nebuliser	Flow rate (l min^{-1})	MMD (μm)	% less than 5 μm
Hudson	8	4.1	60.3
	10	3.2	70.8
System 22	6	4.2	56.2
	8	2.8	72.4
	10	2.4	77.5
System 22 with Optimist	10	1.1	99.9
Turret	6	3.6	70.4
	8	2.9	76.5
	10	1.6	90.0
Respirgard II	6	2.0	91.4
	7	2.0	91.4

Each result is the mean of duplicate experiments.

TABLE 2

Size characteristics of aerosols produced by atomization of EPC MLVs (mean size 0.9 μm) in jet nebulisers

Nebuliser	Flow rate (l min^{-1})	MMD (μm)	% less than 5 μm
Hudson	8	3.7	65.0
	10	3.2	72.0
System 22	6	4.4	54.8
	8	3.0	70.8
	10	2.2	81.3
System 22 with Optimist	10	1.4	98.8
Turret	6	2.4	83.2
	8	2.2	84.9
	10	1.8	88.3
Respirgard II	6	1.9	92.3
	7	1.5	94.7

Each result is the mean of duplicate experiments.

Low gas flow rates produced large aerosol droplets and high flow rates produced smaller droplets as has been previously described (Clay et al., 1983a). The MMD and size distribution of aerosols however, was not affected by the presence of large EPC MLVs (4.8 μm) (Table 2) or small EPC MLVs (0.9 μm) (Table 3) in the liquid being atomised. Thus, for all the models of nebuliser investigated the aerosol characteristics of the nebulised liposomes was determined by the choice of nebuliser and the flow rate employed rather than the size of the vesicles themselves.

In the volunteer study, the Respirgard II nebuliser was chosen for liposome delivery because it produced aerosols from liposome formulations with MMDs likely to result in alveolar deposition in the human lung (Tables 2 and 3). Measured MMDs were consistently lower than those produced by the Hudson, System 22 or Turret nebu-

TABLE 3

Size characteristics of aerosols produced by atomization of EPC MLVs (mean size 4.8 μm) in jet nebulisers

Nebuliser	Flow rate ($l\ min^{-1}$)	MMD (μm)	% less than 5 μm
Hudson	10	3.5	67.0
System 22	10	3.0	70.8
System 22 with Optimist	10	1.3	99.9
Turret	8	2.2	85.6
Respirgard II	6	1.6	95.0

Each result is the mean of duplicate experiments.

lisers, whilst the rate of aerosol delivery was more rapid from the Respirgard II than the System 22 with Optimist, which produced aerosols of comparable size. Calculations from the deposited radiolabelled DPPC/Chol liposomes in the MLI gave a mean MMAD ($n = 4$) of 1.25 μm and σ_g of 2.7, also indicative that liposomes delivered from this nebuliser have a high probability of penetrating to the peripheral airways.

In vivo deposition and clearance of free and liposome associated ^{99m}Tc-DTPA

Extruded DPPC/Chol (1:1 mole ratio) liposomes were used to study the fate of liposomes in the human lung, as a similar reverse-phase evaporation formulation had previously been reported to be stable to nebulisation (Taylor et al., 1990). DPPC is an obvious choice as the phospholipid, since it is the most prevalent phospholipid in pulmonary surfactant (Reynolds and Wallander, 1989), whilst inclusion of cholesterol generally increases in vivo stability and decreases the release rate of entrapped hydrophilic materials (Kirby et al., 1980). Dilution of the ^{99m}Tc-DTPA containing liposomes resulted in release of 35% of the entrapped material over 24 h. This gives an indication of the potential in vivo release rate. However, such data is of limited value in predicting in vivo behaviour. Studies of liposomal sodium cromoglycate indicated in vivo release from DPPC/Chol reverse-phase evaporation vesicles was significantly faster than determined in vitro (Taylor et al., 1989).

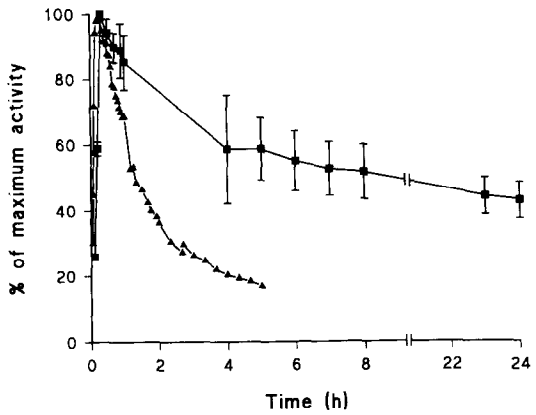

Fig. 1. The deposition and clearance of activity following inhalation of ^{99m}Tc-DTPA in solution (▲) ($n = 1$) and entrapped in DPPC/Chol MLVs (■) ($n = 3$, mean ± S.D.)

Free ^{99m}Tc-DTPA was rapidly cleared from the lungs with activity falling from 100 to 50% over a period of 75 min (Fig. 1). After correcting for radioactive decay, only 16.8% of initially deposited activity remained in the lung region 5 h post-inhalation. No images were acquired after this time as the levels of activity detected were less than twice the background counts. A clearance half-life for free marker of 75 min compares to previously reported figures for inhaled ^{99m}Tc-DTPA in normal subjects of 44 min (Rinderknecht et al., 1980) and 63–70 min (Langford et al., 1986). The principle method for clearance of DTPA solutions from the lungs is by absorption across the pulmonary epithelium into the systemic circulation (Stather et al., 1983). The rate of pulmonary absorption is determined by the physiological condition of the pulmonary epithelium (Barrowcliffe and Jones, 1987) and thus a degree of inter-subject and inter-study variation is to be expected.

The SPET images were reconstructed and analysed in transverse, sagittal, and coronal sections. These indicated a widespread distribution of activity throughout the lungs following inhalation of the liposomal formulation. However, the relatively low levels of radioactivity employed in this study combined with the diffuse nature of airways made more detailed analysis of the images impossible.

At 5 h post-inhalation, 58.5% of the liposome entrapped radioactivity originally deposited in the lung regions still remained compared to 16.8% of free activity at the same time point. At 6 h post-inhalation, 54.8% of the activity remained. Farr et al. (1985) have reported the results of a gamma scintigraphy study in which MLVs and SUVs, with their external lipid surfaces labelled with ^{99m}Tc, were inhaled by healthy volunteers from a Hudson nebuliser. 6-h retentions were 87.5 and 76.8% for the MLVs and SUVs, respectively. A number of factors may be responsible for the differences between these values and the findings of the present study. In the study by Farr et al. (1985) a lipid bound radiolabel was employed. This is likely to remain bound to phospholipid molecules and associate with pulmonary surfactant, even if the integrity of the pulmonary deposited vesicles is compromised during nebulisation or in the airways. An aqueous radiolabel will be lost from vesicles if their integrity is lost or if the marker is released by diffusion across intact bilayers. Released ^{99m}Tc-DTPA will be rapidly absorbed and removed from the pulmonary regions. Additionally, although in this study a nebuliser producing aerosols of a size appropriate for alveolar deposition was employed, the breathing pattern employed by the volunteers was not controlled. Thus it is possible that a greater proportion of the inhaled liposomes were deposited in the conducting airways, from which they are cleared by the mucociliary clearance process, than in the previous study where the nebuliser produced aerosols with larger MMADs (MLVs = 3.7 μm; SUVs = 3.2 μm) but the volunteers inhaled with slow deep breaths.

The levels of radioactivity in the lung regions following inhalation of the liposomal formulation declined rapidly over 4 h and then fell more slowly. The period of rapid clearance of activity may result from clearance of liposomes deposited in the ciliated airways by the mucociliary clearance process. This was confirmed by the appearance of significant levels of activity in the stomach over the first 4 h of the study. Additionally, any ^{99m}Tc-DTPA released as a result of disruption of vesicles during nebulisation, or by diffusion from intact vesicles, will be rapidly removed from the lung regions by absorption. This effect was previously reported when a liposomal formulation of sodium cromoglycate was inhaled by volunteers and plasma levels following pulmonary absorption of drug were monitored (Taylor et al., 1989).

The phase of slow decline of activity in the airways is due to the absorption of activity following release of ^{99m}Tc-DTPA from deposited liposomes. At 24 h post-inhalation 44.7% of the originally pulmonary deposited activity remained in the lungs. This represents the fraction of the radiolabel remaining in intact alveolar deposited liposomes, since in the healthy lung all particles deposited on the conducting airways will have been removed by mucociliary clearance (Newman et al., 1982).

These results indicate the potential of liposomes for drug delivery to the respiratory tract. Sustained release of liposomal contents may be exploited for prolonged local activity of drugs or to achieve prolonged plasma levels of drugs having systemic activity. The formulation developed in this study is stable in the lung and may offer a means whereby the fate of inhaled liposomes in the diseased lung might be explored. Additionally, manipulation of the bilayer composition of the formulation to modulate the release rate of entrapped hydrophilic radiolabel will provide further useful information in the study of inhaled liposomal systems for drug delivery.

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