Distearoyl phosphatidylglycerol liposomes improve surface and transport properties of CF mucus

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Distearoyl phosphatidylglycerol liposomes improve surface and transport properties of CF mucus. S. Girod de Bentzmann, D. Pierrot, C. Fuchey, J-M. Zahm, J-L. Morançais, E. Puchelle. ©ERS Journals Ltd.

ABSTRACT: We have previously shown that a decreased level of phosphatidylglycerol in cystic fibrosis (CF) respiratory mucus is partly responsible for its marked adhesiveness and stickiness, which impair mucus transport, and that distearoyl phosphatidylglycerol (DSPG) was the most efficient form of phosphatidylglycerol in the enhancement of respiratory mucus clearance. The aim of our study was to analyse the effect of distearoyl phosphatidylglycerol liposomes on the transport by cough and cilia of cystic fibrosis respiratory mucus.

The surface and transport properties of mucus were measured: 1) on native cystic fibrosis mucus; 2) on cystic fibrosis mucus complemented with DSPG liposomes at a non-cytotoxic concentration; and 3) on cystic fibrosis mucus complemented with water.

The work of adhesion of cystic fibrosis mucus was significantly decreased by DSPG liposomes, but not by water. For mucociliary transport, the cystic fibrosis mucus was transported at a higher rate with DSPG liposomes and water compared to native cystic fibrosis mucus. The cough clearance of cystic fibrosis respiratory mucus was significantly improved in the presence of DSPG and water, but the effect was more pronounced with DSPG liposomes than with water.

We conclude that the use of DSPG liposomes as a lubricating agent proves to be an interesting therapeutic approach for improving the cough and mucociliary transport in cystic fibrosis patients.
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The respiratory mucus which covers the ciliated respiratory mucosa, is a protective barrier against inhaled exogenous particles and bacteria. The mucus layer is continuously cleared by ciliary beating. In cystic fibrosis (CF), hypersecretion is generally associated with an impairment in mucociliary transport. It has been shown [1–4] that among the various biochemical components of respiratory mucus, there is an increased content of total lipids in CF, which is related to the degree of infection [5]. Generally, there is an imbalance between the different lipid subclasses in CF airway mucus. In particular, total phospholipids are significantly increased in cystic fibrosis [2, 4], as compared with chronic bronchitis or with normal individuals. Among the phospholipids, it has been clearly demonstrated that surface-active fractions, such as phosphatidylcholine or phosphatidylglycerol, are significantly decreased in CF respiratory mucus [2, 3], compared with normal mucus [3]. Conversely, rigidifying phospholipid fractions, such as sphingomyelin, and the ratio phosphatidylserine/phosphatidylinositol are significantly increased in CF disease [2, 3]. This shift towards a rigidifying phospholipid profile induces changes in the physical properties of the respiratory mucus. GALABERT *et al.* [1] demonstrated that the reduced content of phosphatidylglycerol was correlated to a high elastic modulus of CF respiratory mucus. We have

recently reported [2] that the reduced content of phosphatidylglycerol also increases the adhesiveness of CF respiratory mucus. The relative decrease in phosphatidylglycerol of CF respiratory mucus contributes, along with dehydration, to a marked adhesiveness and stickiness responsible for mucus transport impairment and severe bronchial obstruction. We have previously demonstrated [6] that the distearoyl phosphatidylglycerol (DSPG) is the most efficient form of PG in the enhancement of respiratory mucus clearance by cough.

The present study tested the hypothesis that adding liposomes of DSPG to the surface layer of CF mucus and its surrounding fluid would reduce its adhesiveness and accelerate its transport by ciliary activity and by cough.

Materials and methods

Preparation of liposomes

Distearoyl phosphatidylglycerol (DSPG) was provided by Sigma and the high purity of the phospholipid (99%) was controlled by monodimensional thin layer chromatography. Ten milligrams of DSPG were suspended in 10 ml of

distilled water. The preparation was shaken for 2 hours in a water bath at 70°C, at a frequency of 700 oscillations per minute (Oscill 12 Prolabo) in order to form liposomes. The suspension was gently cooled overnight to room temperature and was sterilized by using filtration units (Millipore) with pores of 0.22 µm diameter under aseptic conditions. The liposomes were stored at two different temperatures (4°C and 20°C). The initial concentration of phosphatidyl-glycerol in the suspension was 1.25×10^{-3} M.

Characterization of liposomes

The measurement of size distribution of the liposomes before and after filtration was performed by quasi-elastic light scattering using a Coulter N4 particle analyser (Coultronics). The flocculation tendency or the presence of lipid crystals in the liposome preparation was controlled by observation under phase contrast microscopy. Before filtration, the sizes of the DSPG liposomes were too highly polydispersed to be analysed properly. The filtration process reduced the polydispersity, and after filtration the average diameter of the liposomes was 83.1 ± 1.3 nm and the polydispersity index was 0.23 ± 0.02. The liposome size distribution remained identical for one month, whatever the storage temperature (4 or 20°C). The observation through phase contrast microscopy showed that there was neither crystal formation nor a tendency for flocculation. During this period, the DSPG liposomes remained sterile.

Cytotoxicity assay

The cytotoxicity of the liposomes was controlled on the outgrowth culture of human respiratory surface epithelial cells [7]. Briefly, human respiratory epithelium was obtained from nasal polyps of nonallergic patients undergoing polypectomy due to nasal obstruction. The tissue was cut into small explants (2 mm²), which were seeded onto collagen I matrix. After three days of culture in culture medium RPMI 1640, supplemented with hormones and growth factors, the explant was surrounded by an outgrowth containing ciliated and non-ciliated cells.

Concentrations of DSPG in the liposome suspension were adjusted to 1.25×10^{-3} M, 1.25×10^{-4} M, 2.5×10^{-5} M, 1.25×10^{-5} M and 1.25×10^{-6} M in RPMI 1640. Cultures at day 3 of culture were incubated with the different liposome dilutions for 5 h at 37°C. During the incubation period, the ciliary beat frequency (CBF) was measured by a videomicroscopic technique developed by ZAHM et al. [8].

Cell cultures were then fixed in a 2.5% glutaraldehyde solution in a 0.1 M phosphate buffered saline, postfixed in 1% osmium tetroxide, dehydrated through graded concentrations of ethanol and embedded in agar resin. Semi-thin and thin sections were observed with light and transmission electron microscopy.

Mucus transport

Collection of CF respiratory mucus. Patients suffering from CF (between 8–23 yrs of age, mean 15.5 yrs) were included in this study. They had a forced expiratory volume/forced vital capacity (FEV$_1$/FVC) ranging from 57–106% of the predicted values, and were all colonized by *Pseudomonas aeruginosa*. The respiratory mucus was collected by physiotherapy, and expectoration was protected from salivary contamination by using dental cotton-wool swabs [9].

Surface and transport properties. Surface properties and clearance were analysed on native CF mucus, on CF mucus complemented with DSPG liposomes, and on CF mucus complemented with water.

Surface properties. Two surface parameters were studied: the contact angle, Θ, and the surface tension, Y_{LV}, of respiratory mucus. The measurement of the contact angle was performed as previously described [6, 10]. The contact angle was measured on a 50 µl drop of respiratory mucus, placed on the surface of a glass slide. The contact angle of CF mucus was also measured after adding DSPG liposomes or water: 5 µl of DSPG liposomes or water were deposited onto the glass slide before addition of the CF mucus drop. The glass slides were then placed into a chamber with 100% relative humidity, where the contact angles were measured by an image analysis technique [10].

The surface tension of samples was measured by the platinum ring method [11]. In brief, a fixed platinum ring was put in contact with the sample, which was then moved downwards at a constant speed. A transducer connected to the ring measured the force required to separate the ring and the sample. The measurements of the contact angle, Θ, and of the surface tension, Y_{LV}, of respiratory mucus allowed us to calculate the mucus work of adhesion, Wad, according to the following equation:

$$Wad = Y_{LV} (1 + \cos \Theta)$$

Cough clearance: The experiments were performed using the cough machine developed by KING and co-workers [12–14]. An eight litre tank was used as a reservoir for pressurized gas, and simulated the capacitive function of the lung and the smaller airways. Cough was simulated by opening a solenoïd valve, releasing the pressurized gas through the model trachea connected to the tank. The gas flow through the model trachea was 8 $l \cdot s^{-1}$. The floor of the model trachea was made of the glass slide on which respiratory mucus was placed, in order to obtain the contact angle measurement.

Both the positions of the centre of the mucus drop before cough and of the front edge of mucus after cough were recorded. The distance expressed in millimeters between the two positions represents the mucus cough clearance (CC).

Mucociliary transport: In vitro measurements of the mucus transport velocity were made using the frog palate method [15]. Isolated palates from frogs (*Rana esculenta*) were placed in a plexiglass chamber at a controlled temperature and humidity (30°C and 100% relative humidity). The mucus transport rate was measured by following the displacement of calibrated aluminium discs through a stereomicroscope. The transport rate of the discs progressively

decreased and stopped after 24 h, when the endogenous secretion of mucus was exhausted. At this stage, a drop of mucus (2 µl) taken from the palate of a recently killed frog, was placed on the depleted palate and its transport velocity was measured. The transport velocity of the CF respiratory mucus was then measured and expressed as a mean value of three measurements. The results are expressed in terms of relative transport velocity t_r, corresponding to the ratio of respiratory mucus transport velocity to the control frog mucus transport velocity. The transport velocity of CF respiratory mucus was also measured in the presence of DSPG liposomes or water, by briefly dipping the mucus either into the appropriate dilution of DSPG liposomes or into water, in order to mimic an aerosolization procedure. The quantity of DSPG liposome suspension picked up during this procedure was approximately evaluated by weighing, to 25% of the total mucus volume.

Statistical analysis

The effect of the addition of water or DSPG liposomes on the mucus clearance by cough or ciliary beating, and on the mucus work of adhesion, was studied by a Student's t-test. The ciliary beat frequency (CBF) of respiratory ciliated cells in culture in the presence of DSPG liposomes at various dilutions was compared to the CBF measured in the control cultures by a Student's t-test.

The different values were presented as mean±standard deviation.

Results

Cytotoxicity assay

After 4.5 h of incubation with DSPG liposomes at various dilutions, the CBF was compared to the control values, measured just before adding the liposomes. The CBF was significantly reduced ($p<0.001$) when respiratory cells were incubated with 1.25×10^{-3} M of DSPG and 1.25×10^{-4} M of DSPG in the form of liposomes. Conversely, the CBF was not modified after the incubation period when the respiratory cells had been incubated with DSPG at concentrations of 2.5×10^{-5} M, 1.25×10^{-5} M and 1.25×10^{-6} M. The CBF of the control culture remained stable during incubation in the control defined medium (table 1).

Table 1. – Ciliary beat frequency (mean±SD) of ciliated cells of epithelial cell culture before (T0) and after 4.5 h incubation with various dilutions of distearoyl phosphatidylglycerol (DSPG) and without DSPG (Control).

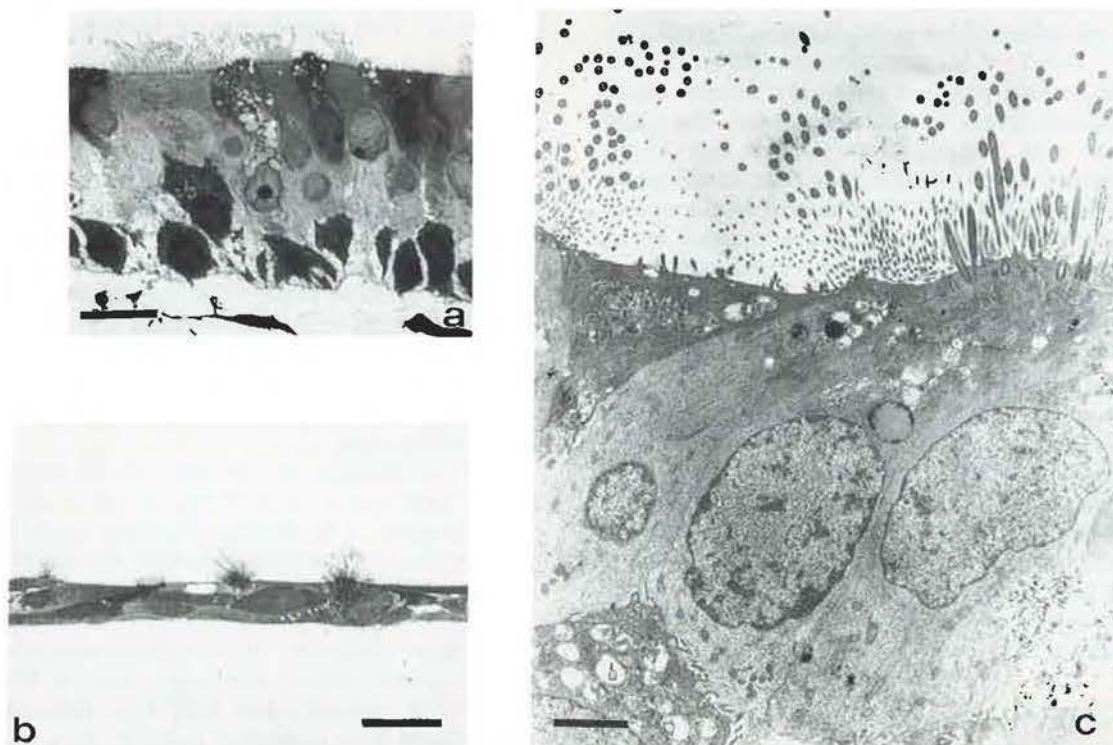| Time incubation | Ciliary beat frequency Hz | | p values |
	T0	4.5 h	
Control	11.7±1.9	11.2±1.7	NS
1.25×10^{-3} M	11.0±1.3	7.9±1.4	<0.001
1.25×10^{-4} M	9.7±1.3	8.3±1.2	<0.001
2.5×10^{-5} M	12.5±1.7	12.4±1.6	NS
1.25×10^{-5} M	11.2±1.9	12.2±2.2	NS
1.25×10^{-6} M	11.3±1.7	10.8±1.7	NS

NS: non signficant.

Fig. 1. – At 2.5×10^{-5} M of DSPG, the respiratory cells in culture are well-preserved and exhibit a morphological aspect in the explant (1a, bar = 10 µm), as well as in the outgrowth (1b, bar = 10 µm), similar to the control culture (not shown). The transmission electron microscopic observation confirmed the well-preserved morphology of the respiratory cells in culture after exposure with DSPG liposomes at a final concentration of 2.5×10^{-5} M of DSPG (1c, bar = 1.5 µm). DSPG: distearoyl phosphatidylglycerol.

The culture was well-preserved after the 5 h incubation period in the presence of 2.5×10^{-5} M of DSPG in the form of liposomes (fig. 1), and the morphological aspect was similar to that observed in the control culture. The concentration of 2.5×10^{-5} M of DSPG in the liposome suspension was selected as the best non-cytotoxic concentration for analysing the effect of the DSPG liposomes on the surface and transport properties of CF respiratory mucus.

Mucus work of adhesion

The surface tension was 72.8 mN·m⁻¹ for water and 63.5 mN·m⁻¹ for DSPG liposomes at 2.5×10^{-5} M in water. The work of adhesion of the native CF mucus (Wad= 134.6±15.8 mN·m⁻¹) was significantly lowered in the presence of DSPG liposomes (Wad=118.1±3.8 mN·m⁻¹, p<0.05) but the addition of water to the CF mucus samples did not change their work of adhesion (Wad=134±3.2 mN·m⁻¹) (fig. 2).
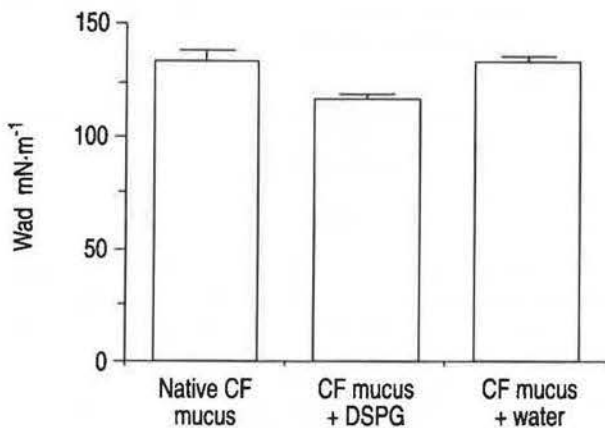

Fig. 2. – Work of adhesion (Wad) of: 1) native CF mucus; 2) CF mucus complemented with DSPG liposomes; and 3) CF mucus complemented with water. The DSPG liposomes significantly decreased the work of adhesion of CF mucus, as compared to native CF mucus (p<0.05), or CF mucus complemented with water (p<0.001). CF: cystic fibrosis; DSPG: distearoyl phosphatidylglycerol.

Mucociliary transport

It is interesting to note that the very low mucociliary transport (t_r=0.56±0.07) of CF respiratory mucus was significantly improved in the presence of water (t_r=0.92± 0.13, p<0.05), as well as of DSPG liposomes (t_r=1.03±0.2, p<0.001) (fig. 3). Nevertheless, the mucociliary transport was not significantly faster with DSPG than with water alone.

Cough clearance

The cough clearance of native CF mucus (CC=22.2±7.6 mm) was also significantly improved with DSPG liposomes (CC=48.6±13.9 mm, p<0.001), and with water (CC=38± 14.5 mm, p<0.05). The effect of DSPG on cough clearance was significantly greater than the effect of water (p<0.05) (fig. 4).
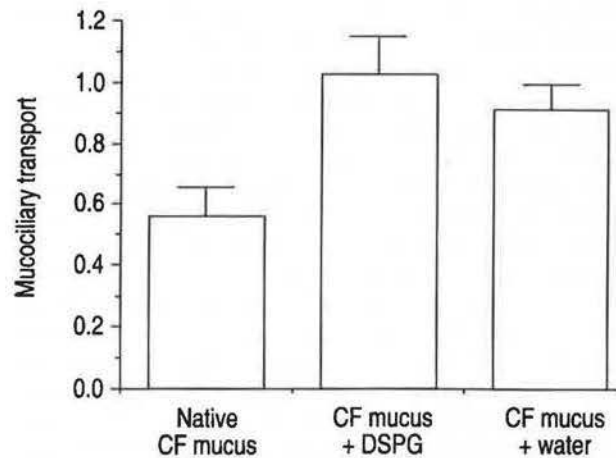

Fig. 3. – Relative mucociliary transport velocity evaluated on the frog palate (t_r) measured by following the displacement of a calibrated aluminium disc deposited on a drop of: 1) native CF mucus; 2) CF mucus complemented with DSPG liposomes; and 3) CF mucus complemented with water. The DSPG liposomes significantly enhanced the mucociliary transport of native CF mucus (p<0.001). For abbreviations see legend to figure 2.
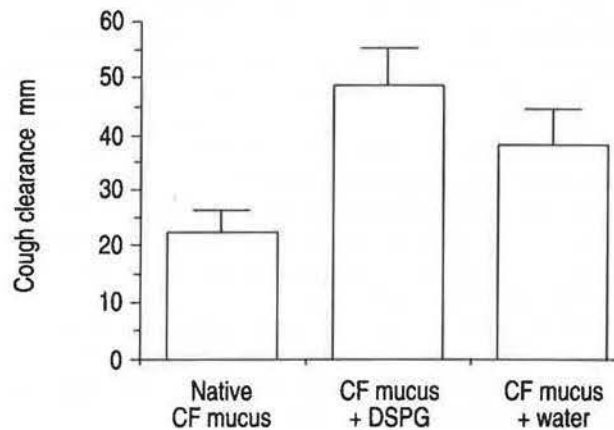

Fig. 4. – In vitro cough clearance in the simulated cough machine of: 1) native CF mucus; 2) CF mucus complemented with DSPG liposomes; and 3) CF mucus complemented with water. The difference was significant between native CF mucus and CF mucus complemented with DSPG liposomes (p<0.001), between native CF mucus and CF mucus complemented with water (p<0.05), between CF mucus complemented with DSPG liposomes and CF mucus complemented with water (p<0.05). For abbreviations see legend to figure 2.

Discussion

We have previously shown that the phospholipid profile of respiratory mucus is altered in cystic fibrosis (CF), with an increased content of the rigidifying phospholipid fractions associated to a decreased content of surface-active fractions [2]. Furthermore, we have shown that the reduced phosphatidylglycerol content induced alterations in the rheological [1], as well as in the surface properties [2], of CF respiratory mucus, which could be responsible for the mucus transport impairment. Supplementing exogenous pulmonary surfactant into the trachea has already been proposed for the treatment and the prevention of the

respiratory distress syndrome in premature infants [16], and in adults [17]. Distearoyl phosphatidylglycerol is, among the phosphatidylglycerol forms, the most efficient component for enhancing mucus cough clearance by decreasing mucus adhesiveness [6]. Stable liposomes of DSPG were prepared without the addition of traditionally used stabilizing agents, such as cholesterol, which possesses rigidifying properties [18–20]. The aqueous phase in which DSPG liposomes were prepared was distilled water, because we were not able to obtain a stable dispersion in an ionic solution. DSPG has a negative electric charge and, in an ionic solution, it is particularly difficult to prepare a stable liposome suspension. This is probably due to the partial neutralization of this electric charge. Nevertheless, the liposomes prepared in water could be subsequently resuspended in a physiological buffer, without any change in their size distribution. A monodisperse and sterile liposome population was obtained by filtration, without the addition of any antibacterial agent. In addition, DSPG, which is a saturated phospholipid, does not become oxidized when exposed to light or air [21]. It was interesting to note that no difference in size was observed, whether liposomes were stored at 4°C or 20°C.

The DSPG liposome suspension adjusted to 2.5×10^{-5} M, which was found to preserve the ciliary activity as well as the morphological aspect of the respiratory epithelial cells in culture, was consequently used for the functional study. The cytotoxic assay was performed directly on a culture model of respiratory epithelium [7], which was not protected by the mucus layer as is the case *in vivo*.

In vivo, DSPG liposomes are more likely to interact directly [22] with the mucus layer rather than with the respiratory epithelium, and could act as a lubricating agent at the mucus-mucosa interface.

In the present work, we added DSPG liposomes or water to CF mucus which is known to be adhesive due to poor hydration [23–28], high osmolarity [29], and lack of surface-active fractions [1–4].

In our *in vitro* experiments, water or liposomes were placed at the interface between the mucus and the substrate, recreating a periciliary sol phase, which significantly enhanced the transport of mucus by cough and by ciliary activity. As emphasized by SCHERER [30], during the cough, the gel mucous layer moves as a rigid slab above the sol phase, which acts as a lubricant between the mucous gel phase and the mucosa. The role of this lubricating layer in allowing airflow to move mucus has already been described by ZAHM *et al.* [31] and GRUENAUER *et al.* [32]. The addition of a surface-active agent, such as DSPG, to this lubricating layer decreases the interfacial forces between the mucosa and the mucus, leading to a better transport of mucus by cough. This lubricating effect of the sol phase is also obvious on the transport of mucus by ciliary activity. During active beating, the cilia penetrate the upper gel phase of the mucociliary system and propel the mucus forward. The strength of this mechanical contact probably depends on the adhesive properties of the gel mucus. Introducing a surface-active agent, such as DSPG, would allow cilia to disengage from mucus at the start of their recovery stroke and, therefore, ciliary beating would be improved, inducing an increase in mucociliary transport.

The dipping procedure, intended to coat the mucus with a surface-active sol phase, was designed to mimic an aerosol application. The alterations in the physical properties of mucus were not due to a dilutional effect or to poor mixing, since the addition of surface-active fractions to water significantly improved this effect. Moreover, ZAHM *et al.* [31] have previously reported that addition of a periciliary phase, as in the present study, did not change either the viscosity or the elastic modulus of the mucus.

The administration of liposomes could be performed by an aerosol or by instillation. When an aerosol of soy phosphatidylcholine liposomes [33] was given to healthy human volunteers to breathe, they suffered no deleterious effects on lung spirometry or arterial oxygen saturation.

The addition of DSPG in an aqueous phase to pathological respiratory mucus, such as cystic fibrosis mucus, could be a very promising therapy for bronchial obstruction due to hypersecretion: distearoyl phosphatidylglycerol liposomes can reduce the adhesiveness of CF respiratory mucus. Our results show that distearoyl phosphatidylglycerol liposomes improve cough clearance and may also acelerate mucociliary transport.

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