Sodium Chloride Increases the Ciliary Transportability of Cystic Fibrosis and Bronchiectasis Sputum on the Mucus-depleted Bovine Trachea

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Abstract

Mucus retention in the lungs is an important feature of several respiratory diseases (Regnis, J.A., M. Robinson, D.L. Bailey, P. Cook, P. Hooper, H.K. Chan, I. Gonda, G. Bautovich, and P.T.P. Bye. 1994. *Am. J. Respir. Crit. Care Med.* 150:66–71 and Currie, D.C., D. Pavia, J.E. Agnew, M.T. Lopez-Vidriero, P.D. Diamond, P.J. Cole, and S.W. Clarke. 1987. *Thorax.* 42:126–130). On the mucus-depleted bovine trachea, the ciliary transport rate of sputum from patients with cystic fibrosis and bronchiectasis of other causes was slow, but the rate was doubled by increasing the sodium chloride content by 90 mM. Increasing the sputum osmolality by inspissation or by the addition of nonelectrolytes had a similar effect. The viscoelasticity of sputum, but not the bovine ciliary beat frequency, was markedly saline dependent over the pathophysiological range. This suggests that low mucus salinity, not (as is generally assumed) its underhydration, contributes to its retention in bronchiectasis due to cystic fibrosis and other causes, probably by affecting its rheology. It also indicates how the genetic defect in cystic fibrosis might lead to impaired mucus clearance. Therapies that increase the osmolality of lung mucus might benefit patients with mucus retention. (*J. Clin. Invest.* 1997. 99:9–13.)
Key words: mucus • osmolar concentration • viscosity • mucociliary clearance • expectorants

Introduction

In health, airway surface hygiene is largely maintained by mucociliary clearance. When this mechanism fails to function adequately in the lungs, mucus accumulates and needs to be coughed up as sputum. Retained mucus invites infection, which may cause a vicious circle of events leading to chronic lung inflammation, typically resulting in bronchiectasis (1). We have shown previously that expectorated sputum differs from mucus obtained from healthy lungs in being poorly transported by a ciliated respiratory epithelium (2). This suggests that one reason for the lung mucus retention in both cystic fibrosis (CF)[1] and non-CF bronchiectasis might be that the mucus itself is intrinsically defective, and therefore lacks the vital property of being efficiently cleared by the beating of airway cilia.

It is often stated that clearance of sputum is impaired by its poor hydration (3, 4), and the rationale for some therapeutic approaches has been to increase the hydration of the retained mucus (5). However, the principal polymer components of sputum gels, mucins and DNA, are polyanionic. The concentration of the major ions, sodium and chloride, and possibly other solutes, would therefore be anticipated to influence the degree of hydration and physical properties of such gels (6). Hypotonic sodium (101 and 131 meq/kg and 95 mM) and chloride (75 and 78 meq/kg and 64 mM) concentrations were reported for CF sputum in three studies (7–9). In contrast, uninfected mucus raised by ciliary action at the tracheostomy sites of laryngectomy patients contained 165 meq/kg sodium and 162 meq/kg chloride (7).

It is not clear how the CF mutation causes the characteristic life-shortening lung disease (10). The predominant ion transport abnormality of CF airway epithelia appears to be an increased rate of sodium absorption (11), combined with decreased permeability to chloride ion due to dysfunction of cystic fibrosis transmembrane regulator protein. Therefore, we have investigated the effects of altered sodium chloride concentration on the ciliary transportability and rheology of respiratory mucus and the effect on ciliary transportability of altering sputum osmolality by inspissation or adding nonelectrolytes. Our observations are relevant to the pathogenesis of CF and other diseases in which lung secretions are retained.

Methods

Sputa were obtained under the supervision of a physiotherapist, and samples with visible quantities of saliva were not used. Samples were placed immediately on ice and frozen to below −20°C within 6 h. Evaporation was minimized by using collections of at least 10 ml and ensuring that the containers were nearly full. Bovine tracheal mucus was collected at the larynx of healthy cattle after slaughter.

Part of this work was presented at a meeting of the American Thoracic Society and was published in abstract form (1995. *Am. J. Respir. Crit. Care Med.* 151:A720).

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Received for publication 31 May 1996 and accepted in revised form 29 October 1996.

J. Clin. Invest.

0021-9738/97/01/0009/05 $2.00
Volume 99, Number 1, January 1997, 9–13

1. *Abbreviations used in this paper:* CF, cystic fibrosis; G′, elasticity; G″, viscosity.

The salinity of sputum or bovine tracheal mucus was altered with free access of water by incubating 0.5-ml aliquots of gel for 48 h with 10 ml of PBS (Dulbecco A; Oxoid, Basingstoke, United Kingdom) of varying concentrations, with penicillin 50 U/ml, streptomycin 50 μg/ml (Gibco, Paisley, United Kingdom), and PMSF (Sigma, Poole, United Kingdom) 0.1 mM. The same 16 samples of sputa (from 8 non-CF bronchiectasis patients and 8 samples from 6 CF patients) were used for the experiments illustrated in Figs. 1 and 2.

The salinity of sputum was changed without altering the gel in any other way by adding solid sodium chloride to samples of sputum. Preliminary experiments showed that crystals of a colored salt (sodium dichromate) dissolved and diffused fully through 2 cm of sputum gel after an overnight incubation at 4°C without the need for mixing. Paired aliquots, each ~ 0.5 ml, were prepared from sputum samples from 15 CF patients and 15 patients with non-CF bronchiectasis. Sodium chloride was added to one aliquot to increase its concentration by 0.5% wt/wt (~ 90 mM); the other aliquot was untreated. Both aliquots were incubated overnight at 4°C. We have shown previously (12) that such incubation does not alter the transportability of sputum. Addition of sufficient sodium chloride to increase its concentration by 90 mM was chosen because this was the approximate difference reported by Matthews et al. (7) between the saline concentration of uninfected mucus and that of CF sputum. Similar experiments were performed in which glucose, mannitol, and urea were added to five sputa (two from CF patients and three from non-CF bronchiectasis) to a final concentration of 0.1 M.

The effect of sputum inspissation was assessed with 12 sputum samples, from 5 CF patients and 7 non-CF patients. Each sample was divided into two aliquots of ~ 1 ml. One aliquot was placed in a shallow dish under a current of air at room temperature and gently stirred at intervals for ~ 2 h until 40–60% of its original weight was lost. The control aliquot was kept in a sealed container.

The ciliary transportability of mucus samples was assayed on 8 cm × 3 cm explants of bovine trachea as described previously (2). Briefly, the explants were depleted of endogenous mucus by prolonged incubation at 37°C followed by repeated application of 0.5 ml bovine mucus. The transport rate of applied mucus was measured at 37°C by observing its movement by eye using a millimeter scale. The transportability index of a mucus sample was its transport rate expressed as a percentage of that of the internal standard, which was bovine tracheal mucus.

For ion measurements, sputum (0.2–0.5 ml) was diluted 1:20 (wt/vol) in isotonic 50 mM Tris 125 mM magnesium sulphate adjusted to pH 7.3 with sulfuric acid and then centrifuged to remove cells and debris. This dilution allowed best use of the limited quantities of sputum and minimized the potential for evaporation. Sodium was measured using an ion-selective minielectrode (Fluka, Gillingham, United Kingdom) and chloride was assayed with the Sigma 461-M mercuric thiocyanate reagent.

Osmolality was measured by freezing point depression using a Roebling microosmometer (type 13). Sufficient sputum for this assay was available from 12 of the CF samples and 12 of the non-CF bronchiectasis samples.

The elasticity (G′) and viscosity (G″) of the 16 sputum samples equilibrated in excess PBS of different concentrations were measured using a CarriMed CS rheometer having 4-cm-diameter parallel plate geometry, with a gap of 0.4 mm and a frequency of 0.16 Hz. Duplicate measurements were made for each sputum and saline concentration, and the mean was calculated.

The beat frequency of bovine tracheal cilia was measured in PBS of varying concentrations. Epithelium was removed from the cartilage of a segment of bovine trachea, dissected into pieces ~ 5 mm square, and kept in 300 mosM PBS. Samples were then placed into warmed PBS of different salinities, and the beating frequency of the cilia visible at the edge of the tissue was measured photometrically at 10 different areas after 15 min of incubation at 37°C (13). The microscopist was ignorant of the ionic strength of the wet mount.

Statistical calculations were performed using the Minitab® program.

Results

Fig. 1 shows the relationship between the concentration of buffered saline (present in excess) and ciliary transportability for bovine tracheal mucus and sputum. In the range of 300 to 500 mosM saline, bovine mucus and sputum were both transported rapidly on the mucus-depleted bovine trachea in vitro. In contrast to the results for isotonic and hypertonic saline solutions, increasing the hydration of sputum by incubation in hypotonic solutions resulted in lower rates of ciliary transport. No difference was apparent between the CF and the non-CF sputa. In these experiments solutes were permitted to flow out of, as well as into, the gels. Therefore, the endogenous solutes were almost completely replaced by saline. Free flow of water could also occur.

The influence of sodium chloride content on sputum transportability was also measured in experiments in which solid sodium chloride was dissolved to 0.5% in the sputum gel. In this design, no change in the water content could occur, and all the products of infection and inflammation were retained. For the 15 CF sputa the mean transportability index (±SEM) increased from 33±3 for neat sputum to 57±5 after salination, $P = 0.001$ (Wilcoxon signed rank test). For the 15 non-CF bronchiectasis sputa the respective values were 26±3 and 58±5, $P = 0.001$. Therefore, the untreated sputa were transported poorly by the ciliary escalator, as reported previously (2). All but one of the sputum samples were better transported by respiratory cilia after adding sodium chloride, by a factor of approximately two, $P < 0.0001$ overall.

The sodium and chloride values (meq/kg, mean±SD) for the 15 CF sputa were 89±24 and 99±25, respectively. For the 15 non-CF bronchiectasis sputa, the respective values were 113±26 and 101±21. Therefore, varying the sodium chloride concentration of sputum in the pathophysiological range profoundly altered the speed at which it was carried by the ciliary

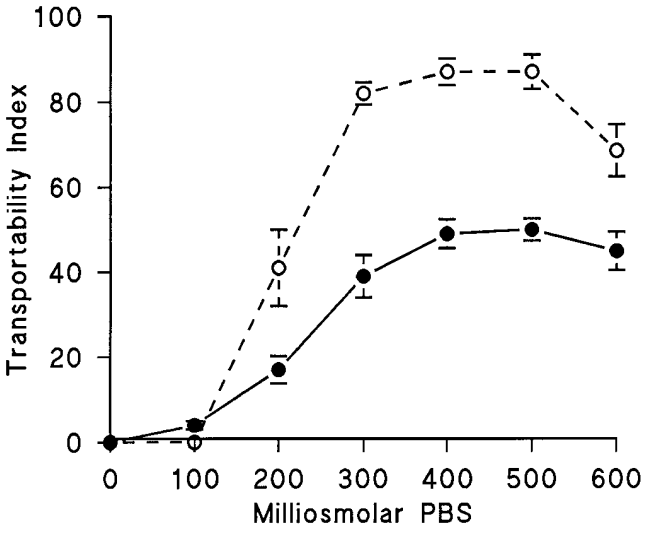

Figure 1. The ciliary transportability of 16 sputum samples (8 CF, 8 non-CF bronchiectasis) (*filled circles*) and 5 bovine tracheal mucus samples (*open circles*) after incubation in excess PBS of varying osmolar concentrations. Bars indicate SEM. $P < 0.006$ for the 200 vs. 400 mosM comparison, for both bovine mucus and bronchiectasis sputum, Wilcoxon signed rank test.

Table I. Beat Frequency of Bovine Tracheal Epithelial Cells in PBS of Varying Concentrations

PBS concentration	Ciliary beat frequency
mosM	*Hz (SEM)*
0	0
100	6.3 (0.2)
200	11.8 (0.3)
300	14.6 (0.5)
400	12.4 (0.3)
500	11.8 (0.4)
600	13.3 (0.4)

Epithelium was removed from cartilage, and the beat frequency was measured photometrically at 37°C after 15 min of incubation in the stated PBS concentration.

escalator. These effects were observed even though the water contents of the sputa were unaltered.

Addition of the nonelectrolytes glucose, mannitol, and urea in solid form to a final concentration of 0.1 M caused the transportability index of sputum (mean±SEM) to increase in every case, from 26±2.9 to 59±4.4, $P < 0.001$ Wilcoxon signed rank test. Inspissation of the 12 sputum samples caused 11 to be more rapidly transported, the transportability index increasing from 28±4.0 to 57±4.0, $P = 0.004$.

The mean osmolality of the CF sputa was 262 mosM/kg (range 171–339, SEM 15). That of non-CF bronchiectasis sputum was 240 (range 177–300, SEM 9).

The ciliary beat frequency of bovine tracheal cilia varied little over the range of 200 to 600 mosM saline (Table I). However, the G′ and G′′ of sputum was markedly dependent on saline concentration (Fig. 2). Increasing the salinity altered the visible appearance of the gel and lowered the values of both G′ and G′′. However, the absolute values of G′ and G′′ did not appear to be of prime importance to transportability, because there were > 100-fold differences in these values between different sputa that were transported at similar rates on the bovine trachea. Nevertheless, every sputum became less viscous and less elastic with increasing salinity. There was no apparent difference between CF and non-CF sputa in their viscoelastic response to salinity.

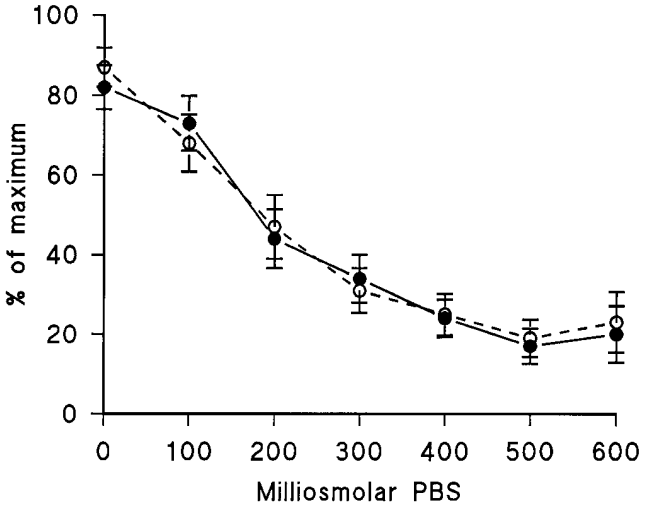

Figure 2. G′(*filled circles*) and G′′(*open circles*) of the same 16 sputa used in Fig. 1 after incubation at 4°C in excess PBS of different concentrations. The values of G′ and G′′ are expressed as a percentage of the maximum recorded measurement for each sputum. Bars indicate SEM. $P < 0.005$ for the 200 vs. 400 mosM comparison, for both G′ and G′′, Wilcoxon signed rank test.

Discussion

Adding sodium chloride to sputum enhances its transportability on a mammalian ciliated respiratory epithelium, but incubating sputum or respiratory mucus in hypotonic solutions had a detrimental effect. Mucus equilibrated with 300–500 mosM saline was maximally transported. It was poorly transported when its salinity was 200 mosM, and almost stationary when equilibrated in 100 mosM saline or water. The ciliary transportability of sputum was also increased by increasing the osmolality of the gel using other means, such as adding the nonelectrolytes glucose, mannitol, and urea, or causing water loss by evaporation.

The efficiency of mucociliary clearance depends on both the characteristics of the mucus and the beating of the cilia. We have shown, as others have (14), that the viscoelasticity of respiratory mucus is saline dependent in the pathophysiological range. In contrast, the ciliary beating frequency of the bovine model system was little changed over the saline concentration of interest. Human bronchial ciliary beat frequency has also been reported to be unaltered in the range of 160 to 400 mosM saline (15). All the sputa had saline contents in this range, both before and after the addition of sodium chloride. Therefore, altered salinity appears to influence the rheology and the ciliary transportability of mucus, but has little effect on the ciliary beat frequency at the concentrations present in sputum. However, the exact rheological determinants of optimum ciliary transportability on the bovine trachea remain to be defined.

New insights into the pathogenesis of mucus retention in CF may be derived from this data. The composition of the airway surface liquid, both in health and in the early stages of CF when bronchiectasis is developing, is unknown. The only analyses thus far reported used methods likely to have perturbed its composition significantly. One estimate, using the application of filter paper to the tracheae of three CF individuals yielded salinity values higher than were found in normal individuals (16). However, the potassium concentration was high compared with that in laryngectomy mucus, raising the possibility of cell damage. The technique involved saturating filter paper many times thicker than the normal airway surface liquid, which could have induced liquid secretion from a surface which is normally absorptive.

The saline dependence of antibacterial defenses presents an interesting contrast. Human neutrophil bactericidal functions against *Pseudomonas aeruginosa*, a common pathogen in CF, were less efficient at low salinity. Bactericidal activity at 62 mM sodium was only 29% of that at 124 mM (17). In contrast, bacterial killing by a defensin-like substance appeared to require a low sodium chloride concentration, and the hypothesis was advanced that in CF a high airway surface salinity led to poor antibacterial activity in this first line defense system (18).

However, the predominant ion transport abnormality of the CF airway appears to be excessive absorption of sodium

(19). This would be predicted, if anything, to lower the airway surface salinity. It has also been thought that this would lead to airway surface dehydration, but this is believed to be effectively prevented by the capillary pressure generated at the air interface by the dense meshwork of cilia and microvilli (20). Therefore, the increased sodium absorption in the CF airway would simply lower the airway surface salinity, which we have shown would have a detrimental effect on mucus transportability. The individual with CF may have poor mucociliary clearance from the moment of birth, because of low airway surface liquid salinity and the consequent defective mucus rheology.

The inverse relationship between sputum salinity and viscoelasticity might also render CF mucus poorly clearable by coughing. We suggest that the low salinity of CF mucus from birth might lead to high viscoelasticity and impaired clearability by this mechanism also. This would explain the well documented difference in functional status and prognosis between those with CF and those with immotile cilia who have no lung mucociliary clearance but generally better health (21). These patients, although often developing bronchiectasis and *Pseudomonas* colonization, have constitutionally normal ion transport, and therefore would have normal mucus salinity and cough clearability during the crucial early period of life. It is of interest that aerosolized UTP, a chloride secretagogue, appears to improve the cough clearability of mucus in patients with primary ciliary dyskinesia (22).

It is noteworthy that the sodium and chloride concentrations in non-CF bronchiectasis sputum, though higher than exist in CF sputum, are also below those found in plasma, and below the optimum for ciliary transportability.

Our results help provide a rationale for the clinical use of nebulized hypertonic saline which has been used for decades as an aid to expectoration. It is one of the few substances which has been shown to increase tracheobronchial clearance in vivo (23–25), and it improves spirometry and subjective wellbeing in subjects with CF (26). Its mechanism of action has hitherto been a matter for speculation. We suggest that it may work by directly increasing the salinity of the retained secretions, in particular the gel surface, where improved effectiveness of interactions with cilia may result in increased ciliary clearance.

Our data may also help to explain the beneficial effects of other therapeutic interventions. Nebulized amiloride, a sodium channel blocker, has been used in several studies in CF, with variable clinical results (5, 27, 28). The original rationale for its use was the expectation that it might increase airway surface hydration (5), but the drug appears to increase the sodium content of the sputum without in fact altering its hydration (9, 27). Our data indicate that this would have a beneficial effect on mucociliary transport, as was observed in one of the clinical trials (27). Aerosolized UTP has several actions on respiratory epithelium, including enhanced chloride and mucus secretion, and stimulation of cilia. It accelerates tracheobronchial (mainly mucociliary) clearance in normal individuals and in patients with CF (29).

Mucus retention in the lungs is common, distressing, and arguably the most important factor in maintaining the vicious circle of respiratory damage observed in chronic lung infection due to CF and other more common respiratory diseases such as chronic bronchitis and bronchiectasis. Our work suggests that therapies targeted at increasing the osmolality rather than the water content of the retained mucus may benefit persons suffering lung mucus retention by improving mucociliary clearance. It also provides a link between the ion transport defect of the CF airway and mucus retention, which would be expected to lead to the other lung manifestations of the disease.

Acknowledgments

We wish to thank the Royal Brompton Hospital Physiotherapy Department for their help in obtaining sputum samples, Dr. K. Gaber and Mr. S. Perinpanayagam for technical assistance, and Dr. K. MacLeod for help with the ion-selective electrode. We thank Prof. C. Marriott and Mr. S. Ingham for assistance with rheometry, and Dr. R. Wilson and Prof. P. Quinton for reading the manuscript and offering helpful criticism. Bovine tracheas were supplied by P.C. Turner & Co.

P.J. Wills was supported by a grant from Bayer.

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