

Unfractionated heparin reduces the elasticity of sputum from patients with cystic fibrosis

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Broughton-Head VJ, Shur J, Carroll MP, Smith JR, Shute JK. Unfractionated heparin reduces the elasticity of sputum from patients with cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 293: L1240–L1249, 2007. First published September 7, 2007; doi:10.1152/ajplung.00206.2007.—Mucus obstruction of the airway in patients with cystic fibrosis (CF) reduces lung function, invites infection, and limits delivery of inhaled drugs including gene therapy vectors to target cells. Not all patients respond to presently available mucolytics, and new approaches are needed. Our objectives were to investigate the *in vitro* effects of unfractionated heparin (UFH) on the morphology and rheology of sputum and the effect of UFH on diffusion of 200-nm nanospheres through sputum from adult CF patients. Confocal laser scanning microscopy was used to image fluorescently stained actin and DNA components of CF sputum, and atomic force microscopy was used to image isolated DNA networks. The viscoelasticity of CF sputum was measured using dynamic oscillatory rheometry. Nanosphere diffusion was measured through CF sputum using a Boyden chamber-based assay. Actin-DNA bundles in CF sputum were disaggregated by UFH at concentrations of 0.1–10 mg/ml, and UFH enhanced the endonuclease activity in sputum from patients on dornase alfa therapy. UFH significantly reduced the elasticity and yield stress, but not the viscosity, of CF sputum from patients not receiving dornase alfa therapy. Heparin dose-dependently significantly increased the diffusion of nanospheres through CF sputum from patients not on dornase alfa therapy from $10.5 \pm 2.5\%$ at baseline to $36.9 \pm 4.4\%$ at 10 mg/ml but was more potent, with maximal effect at 0.1 mg/ml, in patients who were on dornase alfa therapy. Thus the mucoactive properties of UFH indicate its potential as a new therapeutic approach in patients with cystic fibrosis.

mucolysis; rheology; deoxyribonuclease I; drug diffusion; gene therapy

INHERITED DEFECTS IN THE cystic fibrosis transmembrane conductance regulator (CFTR) gene resulting in deficient and/or defective CFTR protein expression in airway epithelial cells lead to the inflammatory lung disease that is associated with most of the morbidity and mortality in patients with cystic fibrosis (CF). The genetic defect appears to be responsible for decreased constitutive secretion of fluid, antimicrobial peptides and anti-proteases from submucosal gland serous cells (68, 69), dehydration of the airway periciliary liquid layer (5), and an intrinsic inflammatory state in the airways (19, 39, 63). The failure of normal innate defenses (5, 68, 69) initiates infection, and most patients are colonized with bacteria from an early age (9, 15, 37). This stimulates an exaggerated inflammatory response, and the abnormally high numbers of neutrophils

present in the CF airway in the absence of infection increase with infection to a level exceeding a normal response (35).

It is hypothesized that mucins secreted normally into the CF airway contribute to the abnormal physical properties of CF sputum (40). However, mucins are relatively depleted in sputum from CF patients with stable lung disease, which was hypothesized to be due to defective secretion (20) but may partly be the result of bacterial and/or leukocyte protease and glycosidase activity (25, 45, 48). In contrast, during pulmonary exacerbation, mucins MUC5AC and MUC5B are increased in airway secretions (21), although a separate study indicated that these are also relatively degraded (49). CF mucus may not be inherently abnormal (29, 49), and, for example, the viscoelasticity of mucoid CF airway secretions is not different from normal induced sputum (50). Although viscoelasticity increases with purulence, it is not higher in CF when compared with purulent samples from other inflammatory airway diseases (10, 46). The viscoelasticity of purulent CF secretions is primarily due to high concentrations of DNA (33) and actin (62) released from millions of necrotic neutrophils accumulating in the airways. Polymeric F-actin is present at concentrations of 0.1–5 mg/ml (62), and extracellular DNA concentrations of 0.6 (0–9.5) mg/ml have been reported (6), which increase with infective exacerbation (56). In all cases, most of the DNA is of human and not bacterial origin (32). Actin polymerizing in the presence of DNA forms bundles containing both DNA and actin (54). Actin-DNA bundles are present in CF sputum and contribute to its viscoelasticity (54, 60).

Highly viscoelastic secretions are removed with difficulty by both mucociliary clearance and cough mechanisms of airway clearance (30, 42). Depletion of the airway surface liquid layer in CF promotes the formation of adhesive mucus plaques that, together with ciliary damage mediated by bacterial toxins and neutrophil elastase, limit mucociliary clearance (4, 44). Thus clearance of secretions by cough becomes important. However, because CF sputum is a viscous material dominated by elastic properties (36), cough clearance is also limited (27, 42), and it was recently reported that sputum cough transportability is inversely related to DNA concentration in CF sputum (26).

Dornase alfa (Pulmozyme) is a mucolytic that reduces the viscoelasticity of CF sputum both *in vitro* (53) and *in vivo* (52) by cleaving DNA and altering the physical characteristics of sputum. However, dornase alfa does not improve lung function in all patients (14, 16), and there is a need to develop novel mucolytic therapies with the goal of improving lung function and delivery of inhaled drugs, including gene therapy vectors,

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to the bronchial epithelium (30). Alternative approaches include the use of inhaled osmotic agents such as hypertonic saline and mannitol (17) as mucomodulators that, by setting up osmotic gradients, improve periciliary fluid hydration and mucus clearance. Previous studies *in vitro* have indicated that polyanionic molecules may be effective mucoactive agents that thin sputum by disrupting DNA-actin polymer interactions (59). In this study, we examined the effect of polyanionic unfractionated heparin on sputum morphology and rheology and diffusion of 200-nm nanospheres through CF sputum. Some of these results have been published in abstract form (7).

METHODS

Sputum Samples

Spontaneously expectorated sputum samples were collected in sterile hermetically sealed containers from adult CF patients with severe lung disease within 24–48 h of their attending Southampton General Hospital (Southampton, UK) for respiratory exacerbation; samples were frozen at -80°C . Freezing followed by a single thawing step allows for accurate and reproducible analysis of sputum rheology (12, 47). Local research ethics committee approval was obtained for the study.

Patients

Samples for the morphological analysis of sputum were from adult patients ($n = 9$, 2 male) not receiving dornase alfa therapy [age 33.1 ± 2.6 yr, forced expiratory volume in 1 s (FEV1) $41.8 \pm 6.0\%$ predicted]. Samples for the rheological studies were from eight adult patients (3 male) not receiving dornase alfa therapy (age 25.6 ± 1.4 yr, FEV1 $38.9 \pm 6.0\%$ predicted) and seven adult patients (3 male) receiving dornase alfa therapy (age 23.14 ± 2.96 yr, FEV1 $35.7 \pm 4.5\%$ predicted). Samples were collected 2–4 h after dornase alfa therapy.

Sputum Morphology

Sample preparation. Whole nonhomogenized sputum samples (~ 100 mg) were treated on a 10% vol/wt basis with phosphate-buffered saline (PBS) with calcium and magnesium (Invitrogen, Paisley, UK), bovine pancreatic DNase I (Sigma, Poole, UK) at a final concentration of $2.9 \mu\text{g/ml}$ (a concentration achieved clinically; Ref. 55), or unfractionated heparin (UFH) sodium salt from porcine intestinal mucosa with a molecular weight of 13,500–15,000 and an anticoagulant activity of 168 IU/mg (Merck Biosciences, Nottingham, UK) to give final concentrations of 0.1, 1.0, and 10.0 mg/ml and incubated at 37°C for 2 h in a humidified chamber.

DNA and actin were stained simultaneously with $10 \mu\text{l}$ of YOYO-1 ($1 \mu\text{M}$) and $20 \mu\text{l}$ of rhodamine phalloidin ($6.6 \mu\text{M}$) (Molecular Probes) for 20 min at room temperature.

Confocal laser scanning microscopy. Samples were visualized using a Carl-Zeiss laser-scanning LSM 510 system (Carl Zeiss, Jena, Germany) with dual excitation at 488 and 543 nm. The percent area of red, green, and yellow staining corresponding to actin, DNA, and co-localization, respectively, was quantified in five random fields of view for each of the confocal laser scanning microscopy (CLSM) images using an image analysis software program written in Visual Basic.

Sputum Rheology

Effect of heparin on rheological properties of CF sputum. Sputum was thawed at room temperature and gently homogenized 10 times with a 5-ml syringe (Whatman, Kent, UK), and 1-g aliquots were treated with UFH in PBS without Ca-Mg on a 10% vol/wt basis to give final heparin concentrations of 0, 1, and 10 mg/ml and incubated for 1 h at 37°C before rheological analysis.

Validation of sputum homogenization. The homogenization procedure was validated by comparing parameters obtained for three portions of a homogenized sample with three nonhomogenized portions of the same sample.

Rheological analysis. Rheological analysis was performed using an AR 2000 controlled-stress rheometer (TA Instruments, Leatherhead, Surrey, UK). A 6-cm acrylic parallel plate geometry at 20°C was used. A solvent trap containing distilled water prevented dehydration of samples during rheological analysis. The sputum sample (0.71 g) was loaded onto the rheometer with a gap width of $250 \mu\text{m}$ between the plates and equilibrated for 1 h to allow relaxation to the original gel structure. Initial experiments were performed in dynamic oscillatory mode, which is nondestructive. This included a torque sweep (0.001 – 10 Pa) to determine the linear viscoelastic region, which was 0.001 – 0.01 Pa in the case of every sample tested. A frequency sweep (0.1 – 10 Hz) was performed at 0.01 Pa, and the rheological parameters of elasticity (G'), dynamic viscosity (η'), and tan delta were determined in the linear viscoelastic region of all samples using Rheology Advantage data analysis software (TA Instruments) for a range of oscillation frequencies (0.16, 0.3, 2, 5, and 9 Hz). A flow test was performed last, as this is a destructive test from which the sample does not recover. In this test, the shear stress on the sample was increased from 0.005 to 10 Pa, and 20 stress values within this range were tested. The corresponding viscosity was measured for 10 s at each stress, and the yield stress of the sputum sample was determined.

Treatment of Sputum from CF Patients with Heparin for the Extraction of DNA

Whole nonhomogenized CF sputum samples were treated 1:1 (wt/vol) with PBS or UFH to give final concentrations of 0, 0.1, 1, or 10 mg/ml heparin. Samples were vortexed and incubated for 1 h at 37°C with shaking before the extraction of DNA.

DNA Extraction from Sputum

Following the treatment of CF sputum, samples were centrifuged at $20,000 g$ for 20 min. Supernatants were removed ($100 \mu\text{l}$), mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen), and centrifuged at $9,000 g$ for 5 min. The upper aqueous phase was removed and added to a second volume of phenol-chloroform-isoamyl alcohol (25:24:1) and treated as before. The final aqueous layer was removed and added to two volumes of absolute ethanol. This was gently mixed and centrifuged at $9,000 g$ for 1 min. The resulting pellet was dried in a vacuum evaporator before being dissolved in 10 mM Tris buffer, pH 8.5 ($25 \mu\text{l}$), at 56°C . To each sample ($25 \mu\text{l}$) were added $2.5 \mu\text{l}$ of $10\times$ loading dye (50% glycerol, $10\times$ TBE buffer, 1% bromophenol blue), and $15 \mu\text{l}$ were loaded onto an agarose gel. A DNA standard digest 100-bp ladder ($5 \mu\text{l}$) to which had been added $0.5 \mu\text{l}$ of $10\times$ loading dye was added to marker lanes.

Agarose Gel Electrophoresis

A 2% agarose gel was prepared using 2 g of agarose in 100 ml of TBE buffer (16.2 g of Tris, 4.63 g of boric acid, 0.95 g of EDTA in 1 liter of ultra-high-quality water, pH 8.8) containing $1 \mu\text{g/ml}$ ethidium bromide. The gel was submerged in TBE buffer containing $1 \mu\text{g/ml}$ ethidium bromide. The samples were electrophoresed at 80 V for ~ 1 h. Imaging was performed using a UV transilluminator, and the extent of DNA degradation was determined using a Quantiscan analysis package (Biosoft, Cambridge, UK).

Atomic Force Microscopy

Highly polymerized calf thymus DNA (Amersham Biosciences, Bucks, UK) ($100 \mu\text{g/ml}$ in water) was treated with bovine pancreatic DNase I (Sigma) ($2.9 \mu\text{g/ml}$) and heparin (0, 1, 10, $100 \mu\text{g/ml}$) in the presence of magnesium sulfate (1.2 mM) for 1 h at 37°C . Samples ($100 \mu\text{l}$) were dialyzed in Ultrafree-MC centrifugal 5-kDa filter units

(Fisher Scientific, Loughborough, UK) and resuspended in 100 μl of deionized H_2O , and 10 μl were added to freshly cleaved ruby muscovite mica (Agar Scientific, Stansted, UK). Samples were dried by evaporation, and contact mode atomic force microscopy (AFM) topography imaging was performed in air at 25°C using a TopoMetrix TMX2000 Scanning Probe Microscope (Veeco Instruments, Santa Clara, CA), as previously described (8).

Barrier Assay

This assay is based on our recent description of a simple assay to measure the diffusion of 200-nm nanospheres, to model the size of gene therapy vectors, through CF sputum (8). A 48-well micro-Boyden chamber (Neuro Probe) was assembled, and the upper and lower compartments were separated by a polycarbonate filter (8- μm pore size) (Whatman, Kent, UK). The lower compartment (50 μl) contained PBS. CF sputum was gently homogenized with a 2.5-ml syringe (Whatman, Kent, UK) (10 strokes) and divided into aliquots (0.1–0.2 g). We previously reported (8) that this homogenization procedure does not affect sputum pore size. PBS or UFH was added to sputum aliquots on a 10% vol/wt basis. Treated samples were mixed with carboxylate-modified FITC-nanospheres (200 nm in diameter) (Molecular Probes, Paisley, UK) in a 1:1 (vol/vol) ratio before addition of the sputum (20 μl , which gives a depth of 2.8 mm) to the upper well of the micro-Boyden chamber. The chamber was centrifuged briefly at 1,000 rpm (200 g) to remove air bubbles from below the sputum layer. Following incubation of the chamber for 4 h at 37°C, the fluorescence (excitation 485 nm, emission 535 nm) of the solution in the lower well was measured on a Victor plate reader (Wallac/PerkinElmer, Boston, MA).

Statistical Analysis

The Shapiro-Wilk test for normality indicated that data for sputa were not normally distributed. Data were compared with either a nonparametric Kruskal-Wallis test or a Friedman test, followed by a Dunnett's multiple comparison post hoc test, where $P < 0.05$ was the minimum accepted level of significance.

RESULTS

CLSM confirmed the presence of actin-DNA bundles in CF sputum, as previously described (54, 60). The image shown in Fig. 1A is typical of nine different samples analyzed from patients who were not receiving dornase alfa therapy. Actin was partly associated with the DNA fibers in the sample, as indicated by the yellow color, because of co-localization of the polymers, but much was present in a punctuate form that appeared to be cell associated, as previously described (54, 60). Incubation of the samples with UFH induced the dose-dependent disappearance of DNA fibers (Fig. 1, B–D). Control experiments in which highly polymerized calf thymus DNA (20 mg/ml) was stained with YOYO-1 in the presence of 10 mg/ml UFH showed no effect of UFH on fluorescence intensity, indicating that this was not due to heparin interfering with YOYO-1 binding to DNA (Fig. 1, E and F). Quantitative image analysis demonstrated a significant ($P < 0.05$) decrease in the percent area stained for DNA (Fig. 2), with a concomitant increase in the percent area stained for actin, at every concentration of UFH tested.

There is a large degree of heterogeneity within and between individual sputum samples because of the complex nature of the mucus structure. Most previous analyses of CF sputum rheology incorporating oscillatory rheometry have relied on microliter quantities of selected sputum plugs (29), raising the possibility of selection bias in a heterogeneous material like CF

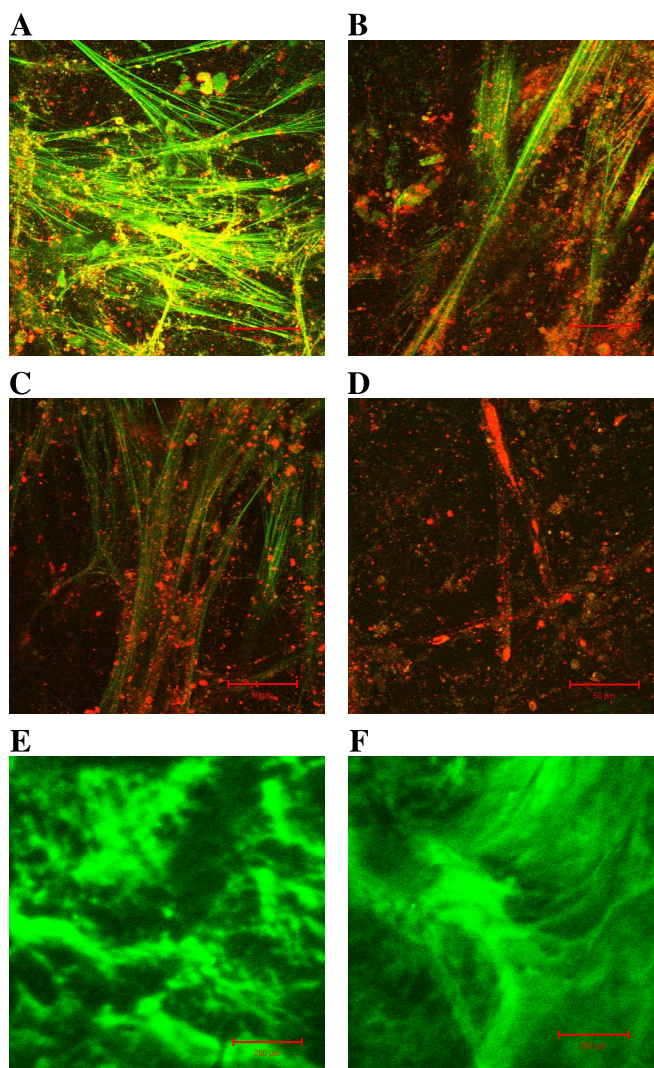


Fig. 1. The effect of unfractionated heparin (UFH) on cystic fibrosis (CF) sputum morphology. A–D: confocal laser scanning microscopy images of whole nonhomogenized CF sputum from a patient not receiving dornase alfa therapy, treated with PBS (A), 0.1 mg/ml UFH (B), 1.0 mg/ml UFH (C), and 10 mg/ml UFH (D) and stained with YOYO-1 for DNA (green) and rhodamine phalloidin for actin (red). The yellow color indicates co-localization of DNA and actin. The scale bar indicates 50 μm . E and F: control staining of calf thymus DNA (20 mg/ml) with YOYO-1 in the absence (E) and presence (F) of UFH (10 mg/ml). The scale bar indicates 200 μm .

sputum. In the present investigation, we analyzed the macro (rather than micro)-rheological properties of CF sputum by analyzing gently homogenized samples instead of selected sputum plugs. Three aliquots from a homogenized sputum sample were compared with three aliquots from the same nonhomogenized sample, and the coefficient of variation among the three aliquots in each of four separate cases was compared (Table 1). The rheological characteristics of gently homogenized sputum specimens were lower than, but not significantly different from, values obtained for selected non-homogenized aliquots from the same sputum samples. Although the lower values of homogenized samples may indicate a degree of shear-induced polymer cleavage, tan delta (the ratio of viscosity to elasticity) values of <1.0 (Table 2) for the untreated homogenized samples indicate that elastic contribu-

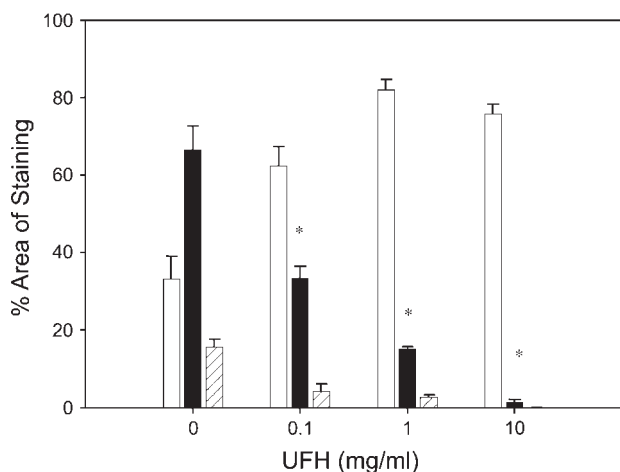


Fig. 2. Quantitative analysis of the percent area of staining contributed by actin (open bars), DNA (black bars), and co-localization of actin and DNA (hatched bars) pre- and posttreatment with UFH. Bars represent means \pm SE; $n = 9$. * $P < 0.05$.

tions continue to dominate sputum rheology, as previously reported (36).

However, the coefficient of variation for measurements of both the storage modulus (G') and dynamic viscosity of non-homogenized samples was significantly ($P < 0.05$) greater than that for homogenized samples. Gently homogenizing sputum, with subsequent recovery of the gel structure over a 1-h period before rheological analysis, therefore results in more reproducible data requiring a smaller number of samples to test the dose-responsive effects of mucolytics.

The effect of UFH (0, 1, 10 mg/ml) on the elasticity, dynamic viscosity, and tan delta (the ratio of viscosity to elasticity) of sputum was tested at a range of frequencies using samples from patients receiving dornase alfa therapy ($n = 7$) and those not on dornase alfa therapy ($n = 8$). The full data set is presented in Table 2. In summary, the rheological parameters of elasticity and dynamic viscosity were lower, and tan delta values were higher, but not significantly different, for samples from patients who were receiving dornase alfa therapy compared with those that were not. Significant effects of UFH on elasticity (Fig. 3) and tan delta (Fig. 4) were recorded only for samples from patients not receiving dornase alfa therapy, and there was no effect of UFH on sputum viscosity at any frequency investigated.

Figure 3 shows the storage modulus (elasticity) values obtained for sputum from non-dornase alfa-treated CF patients over a range of oscillation frequencies. The storage modulus of sputum at 0.16 Hz in the absence of UFH was 5.05 (1.60–7.69) Pa, and treatment of sputum with UFH at a concentration of 1 mg/ml significantly ($P < 0.05$) reduced the storage modulus to 1.86 (0.93–3.75) Pa. There was no significant difference between the values obtained at 1 and 10 mg/ml UFH. A dose-dependent effect of UFH was seen only at 0.3 Hz. Conversely, the addition of UFH to sputum from dornase alfa-treated patients had no significant effect on storage modulus. For sputum from both non-dornase alfa-treated and dornase alfa-treated CF patients, as the oscillation frequency increased, the storage modulus values significantly ($P < 0.001$) increased; however, the trends in the effect of UFH remained consistent across the frequency range (Fig. 3).

The dynamic viscosity values obtained for sputum from non-dornase alfa-treated and dornase alfa-treated CF patients were not significantly different, and UFH had no significant effect at any frequency (Table 2). For sputum from both non-dornase alfa-treated and dornase alfa-treated CF patients, as the oscillation frequency increased, the dynamic viscosity values decreased significantly ($P < 0.001$).

Figure 4 shows the tan delta (ratio of viscosity to elasticity) values obtained for sputum from non-dornase alfa-treated CF patients. At a frequency of 0.16 Hz, the tan delta of sputum in the absence of UFH was 0.31 (0.25–0.36). Treatment of sputum with UFH at a concentration of 1 mg/ml increased the tan delta value to 0.38 (0.31–0.44), but this was not significant. In the presence of UFH at 10 mg/ml, the tan delta value was significantly ($P < 0.05$) increased to 0.38 (0.33–0.60) compared with the absence of UFH. Tan delta for the samples from dornase alfa-treated CF patients was not significantly different from the value obtained for non-dornase alfa-treated patients at any frequency, and UFH had no significant effect.

The yield stress of sputum samples was measured following in vitro treatment with UFH. This was determined using a flow test, in which the shear stress is increased and the corresponding viscosity measured. At lower shear stress, the viscosity remains constant. The yield stress of a material is the applied stress at which the transition from elastic to plastic deformation occurs. On reaching the yield stress, the viscosity of the material drops sharply as it exhibits non-Newtonian pseudo-plastic behavior and is unable to recover from the applied shear stress, i.e., the point at which the material starts to exhibit liquid-like behavior. Figure 5 shows an example of a flow curve obtained for a CF sputum sample in the presence of UFH at 0, 1, and 10 mg/ml, resulting in yield stresses of 14.2, 13.4, and 8.0 Pa, respectively. A summary of the effect of UFH on the yield stress of sputum from non-dornase alfa- and dornase alfa-treated patients is shown in Table 3. The yield stress of non-dornase alfa-treated sputum was greater than the yield stress of dornase alfa-treated sputum; however, the difference was not significant. UFH at a concentration of 10 mg/ml caused a significant ($P < 0.01$) decrease in the yield stress of sputum from non-dornase alfa-treated CF patients. However, UFH had no significant effect on the yield stress of sputum from dornase alfa-treated CF patients.

Table 1. Effect of homogenization on the storage modulus and the dynamic viscosity of CF sputum at a frequency of 0.16 Hz

Sputum Sample	Homogenized	Nonhomogenized
*Storage modulus, Pa (CoV, %)		
1	10.1 \pm 2.0 (34.7%)	123.6 \pm 64.6 (90.5%)
2	7.7 \pm 0.9 (20.5%)	36.6 \pm 17.8 (84.3%)
3	2.3 \pm 0.3 (24.2%)	8.1 \pm 2.0 (42.8%)
4	5.6 \pm 1.0 (30.6%)	25.2 \pm 14.3 (98.4%)
†Dynamic viscosity, Pa.s (CoV, %)		
1	2.6 \pm 0.5 (30.8%)	101.2 \pm 86.9 (148.7%)
2	2.6 \pm 0.3 (20.4%)	20.1 \pm 17.6 (152.2%)
3	0.5 \pm 0.1 (27.6%)	1.7 \pm 0.4 (42.8%)
4	1.4 \pm 0.2 (21.9%)	13.4 \pm 10.9 (140.9%)

Results are expressed as means \pm SE, with coefficient of variation (CoV; in %) in parentheses. CF, cystic fibrosis. *Storage modulus = elasticity (G'). †Dynamic viscosity = η' .

Table 2. Rheological parameters of elasticity, dynamic viscosity, and tan delta of heparin

Frequency, Hz	-Dornase Alfa			+Dornase Alfa		
	Heparin					
	0 mg/ml	1 mg/ml	10 mg/ml	0 mg/ml	1 mg/ml	10 mg/ml
<i>Elasticity, Pa</i>						
0.16	5.05 (1.60–7.69)	1.86 (0.93–3.75)*	1.76 (0.89–11.56)	1.58 (0.64–10.83)	1.80 (0.11–11.64)	2.41 (1.15–7.69)
0.3	5.58 (1.84–8.45)	2.16 (1.04–4.44)	0.42 (0.19–2.26)†	1.79 (0.80–11.59)	2.07 (0.18–12.42)	0.46 (0.23–1.33)
2	7.45 (2.59–10.96)	3.35 (1.44–6.97)*	3.10 (1.53–19.31)	2.45 (1.50–14.18)	3.19 (0.72–15.07)	3.44 (1.84–11.38)
5	8.22 (2.88–12.48)	4.13 (1.54–8.38)*	3.71 (1.88–23.41)	2.86 (2.01–15.55)	3.78 (1.33–16.65)	4.12 (2.12–13.64)
9	8.68 (2.78–13.64)	4.91 (1.40–9.17)*	4.37 (2.32–26.86)	3.45 (2.55–16.26)	4.54 (2.58–17.77)	4.93 (2.40–15.34)
<i>Dynamic viscosity, Pa.s</i>						
0.16	1.42 (0.56–2.07)	0.75 (0.28–1.65)	0.70 (0.32–3.77)	0.63 (0.39–1.97)	0.68 (0.29–2.04)	0.81 (0.39–2.15)
0.3	0.76 (0.32–1.21)	0.46 (0.17–0.99)	0.42 (0.19–2.26)	0.35 (0.26–1.09)	0.39 (0.22–1.13)	0.46 (0.23–1.33)
2	0.13 (0.06–0.26)	0.11 (0.03–0.22)	0.10 (0.04–0.56)	0.08 (0.05–0.21)	0.07 (0.07–0.22)	0.10 (0.04–0.32)
5	0.06 (0.03–0.13)	0.06 (0.02–0.11)	0.05 (0.02–0.30)	0.05 (0.03–0.11)	0.04 (0.04–0.11)	0.05 (0.02–0.18)
9	0.04 (0.02–0.09)	0.05 (0.01–0.08)	0.04 (0.02–0.20)	0.04 (0.02–0.07)	0.04 (0.03–0.07)	0.04 (0.02–0.12)
<i>‡Tan delta</i>						
0.16	0.31 (0.25–0.36)	0.38 (0.31–0.44)	0.38 (0.33–0.60)*	0.38 (0.18–0.65)	0.36 (0.18–2.63)	0.30 (0.21–0.60)
0.3	0.29 (0.24–0.34)	0.38 (0.30–0.42)	0.37 (0.32–0.59)	0.36 (0.18–0.65)	0.39 (0.17–2.29)	0.31 (0.20–0.58)
2	0.29 (0.20–0.33)	0.39 (0.30–0.45)	0.37 (0.33–0.58)*	0.37 (0.19–0.78)	0.43 (0.18–1.26)	0.34 (0.20–0.62)
5	0.31 (0.23–0.38)	0.42 (0.33–0.52)*	0.39 (0.37–0.62)*	0.41 (0.21–0.87)	0.49 (0.20–1.03)	0.40 (0.25–0.72)
9	0.35 (0.26–0.46)	0.51 (0.39–0.55)	0.42 (0.40–0.63)	0.42 (0.24–0.87)	0.61 (0.22–0.87)	0.45 (0.29–0.68)

Results are expressed as median (range). Sputum was collected from non-dornase alfa-treated (-dornase alfa; $n = 8$) and dornase alfa-treated (+dornase alfa; $n = 7$) CF patients and treated with unfractionated heparin (UFH; 0, 1, or 10 mg/ml heparin, as indicated) for 1 h at 37°C. Rheological parameters of elasticity, dynamic viscosity, and tan delta were measured at a range of frequencies. ‡Tan delta = ratio of viscosity to elasticity. * $P < 0.05$ and † $P < 0.01$ compared with no-UFH controls.

Figure 6A shows an agarose gel analyzing the molecular size of DNA in sputum from three CF patients who were not receiving dornase alfa therapy and were treated in vitro with a range of UFH concentrations (0, 0.1, 1, 10 mg/ml). Scanning

densitometry showed that, at all concentrations tested, UFH increased the amount of intact DNA extracted from the samples but had no effect on DNA degradation in these samples. The results shown are representative of six different samples,

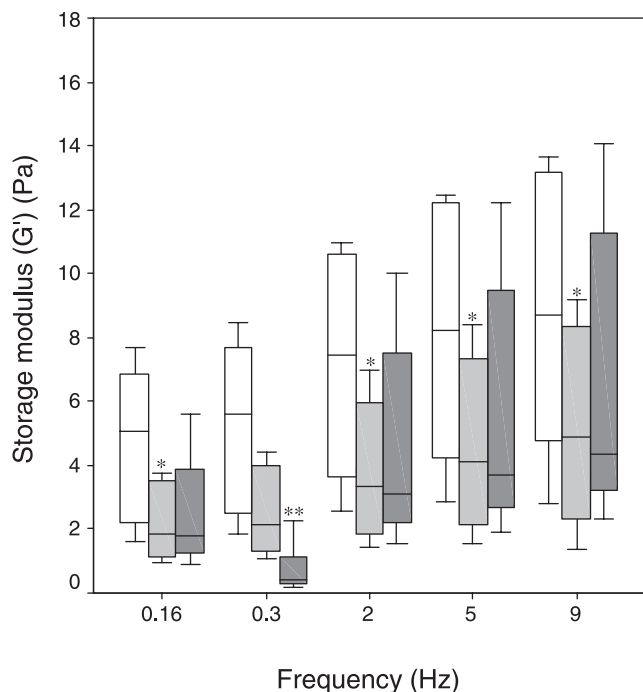


Fig. 3. Effect of UFH on storage modulus (G') of sputum from non-dornase alfa-treated CF patients ($n = 8$). Sputum was collected from non-dornase alfa-treated CF patients, homogenized, mixed with UFH, and analyzed using an AR 2000 controlled-stress rheometer to determine the storage modulus of sputum at a range of frequencies. Open bars, no UFH; light grey bars, 1 mg/ml UFH; dark grey bars, 10 mg/ml UFH. Results are expressed as median (range). * $P < 0.05$ and ** $P < 0.01$ compared with sputum in the absence of UFH.

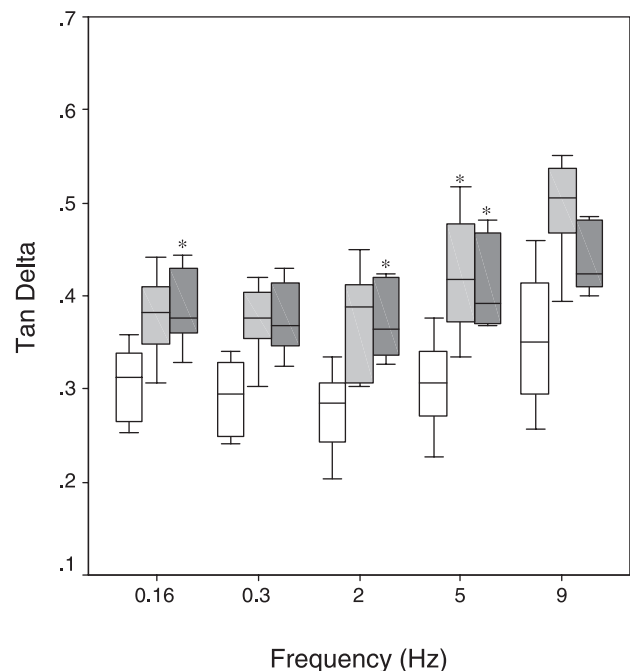


Fig. 4. Effect of UFH on tan delta of sputum from non-dornase alfa-treated CF patients ($n = 8$). Sputum was collected from non-dornase alfa-treated CF patients, homogenized, mixed with UFH, and analyzed using an AR 2000 controlled-stress rheometer to determine the tan delta of sputum at a range of frequencies. Open bars, no UFH; light grey bars, 1 mg/ml UFH; dark grey bars, 10 mg/ml UFH. Results are expressed as median (range). * $P < 0.05$ compared with sputum in the absence of UFH.

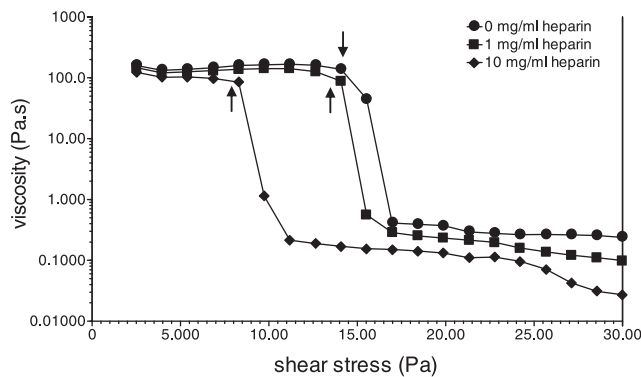


Fig. 5. A flow curve of homogenized CF sputum in the absence and presence of UFH (0, 1, or 10 mg/ml). The final test performed on each sputum sample was a destructive flow test that allowed the yield stress of the sputum to be determined in the presence and absence of UFH. Arrows indicate yield points.

and the amount of DNA extracted >500 base pairs in size was significantly ($P < 0.05$) greater at 0.1 mg/ml UFH ($n = 6$). Figure 6B shows an agarose gel analyzing DNA extracted from sputum samples from two CF patients on dornase alfa therapy. The results are representative of five different samples analyzed. A clear dose-dependent effect of UFH on DNA degradation was observed in all samples. Scanning densitometry showed that UFH at 1 and 10 mg/ml significantly ($P < 0.05$) increased the amount of degraded DNA (<500 base pairs) present.

DNA networks were imaged using AFM to investigate the effect of UFH on bovine DNase I activity. As shown in Fig. 7, highly polymerized calf thymus DNA (100 $\mu\text{g/ml}$) forms a uniform network (Fig. 7A). The addition of DNase at a concentration achieved clinically (2.9 $\mu\text{g/ml}$) (55) appeared to disrupt the DNA network, although DNA hydrolysis appeared negligible (Fig. 7B). However, the addition of DNase together with low concentrations of UFH (1, 10 $\mu\text{g/ml}$) resulted in destruction of the DNA network and dissolution of most of the DNA (Fig. 7, C and D). When DNase was combined with 100 $\mu\text{g/ml}$ UFH, the mica appeared completely bare (Fig. 7E). In control experiments, treatment with 100 $\mu\text{g/ml}$ UFH alone left a dense, but distorted, network of DNA on the mica, indicating that UFH was not simply displacing DNA from the mica (Fig. 7F). In addition, concentrations of UFH from 1 $\mu\text{g/ml}$ to 10 mg/ml, alone, do not degrade isolated calf thymus DNA when analyzed by agarose gel electrophoresis (not shown). The greater potency of UFH in the AFM assay compared with other assays used in this study probably reflects the lower substrate concentration (100 $\mu\text{g/ml}$), which is a concentration of DNA chosen to produce a homogeneous microporous network that can be readily observed in the AFM images.

Table 3. Yield stress of heparin

-Dornase Alfa			+Dornase Alfa		
Heparin					
0 mg/ml	1 mg/ml	10 mg/ml	0 mg/ml	1 mg/ml	10 mg/ml
13.9 (2.3–22.6)	5.2 (1.6–13.4)	1.6 (0.0–9.3)*	5.9 (1.7–15.4)	7.4 (1.6–14.2)	3.2 (2.3–12.7)

Values are for yield stress, in Pa. Results are expressed as median (range). Sputum was collected from non-dornase alfa-treated ($n = 8$) and dornase alfa-treated ($n = 7$) CF patients and treated with UFH (0, 1, or 10 mg/ml heparin, as indicated) for 1 h at 37°C. * $P < 0.05$ compared with no-UFH controls.

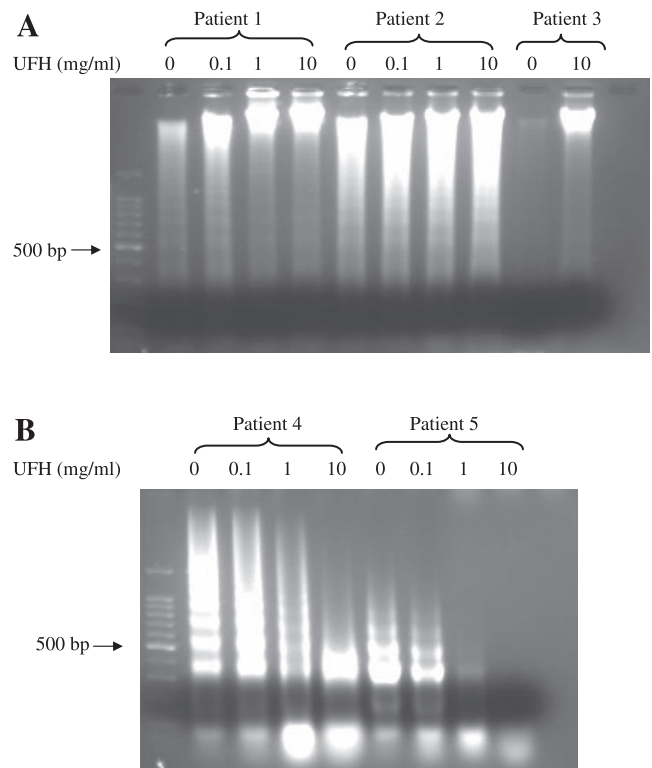


Fig. 6. Effect of UFH on DNA in sputum from non-dornase alfa-treated (A) and dornase alfa-treated (B) CF patients. Patients 1–3 were not treated with dornase alfa; patients 4 and 5 were treated with dornase alfa. Sputum was obtained by spontaneous expectoration, and nonhomogenized sputum was treated with UFH (0, 0.1, 1, or 10 mg/ml) in vitro for 1 h at 37°C. DNA was extracted from sputum supernatants, and samples were subjected to agarose gel electrophoresis.

To investigate the effect of UFH on the barrier properties of CF sputum, sputum from both non-dornase alfa-treated and dornase alfa-treated CF patients was mixed with UFH in vitro, and the transport of nanospheres through the sputum layer was measured. Transport of nanospheres is expressed as a percentage of transport through PBS buffer alone (100%). In the presence of CF sputum from non-dornase alfa-treated patients, nanosphere transport was reduced to $10.5 \pm 2.5\%$ of the PBS control (Fig. 8). UFH concentrations of 0.01 and 0.1 mg/ml had no effect on nanosphere transport, but UFH at 1 and 10 mg/ml significantly ($P < 0.01$) increased nanosphere transport to 26.3 ± 2.1 and $36.9 \pm 4.4\%$, respectively, of the PBS control compared with untreated CF sputum. CF sputum from dornase alfa-treated CF patients reduced nanosphere transport to $10.0 \pm 0.5\%$ of the PBS control (Fig. 8), which was not significantly different from the transport of nanospheres

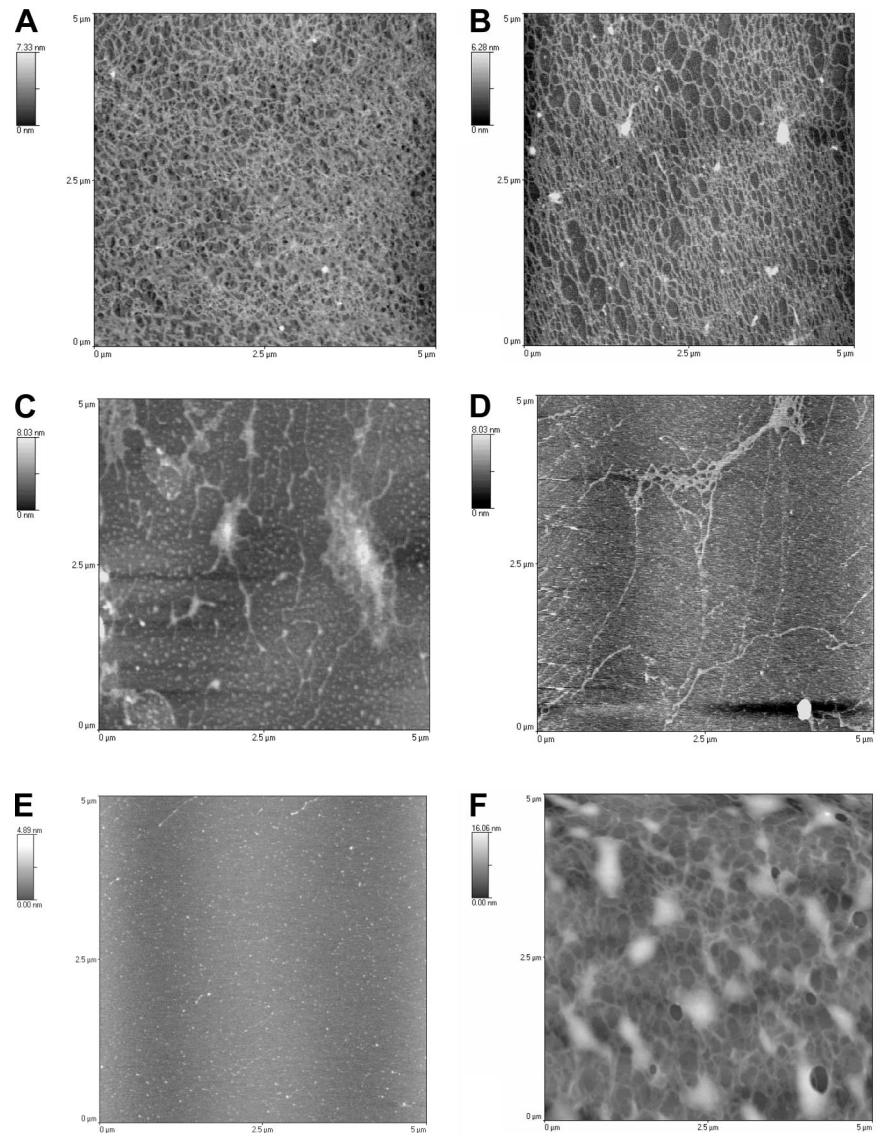


Fig. 7. Effect of UFH on DNase I activity. Typical atomic force microscopy topography images, obtained in air. A: DNA alone at a concentration of 100 $\mu\text{g/ml}$. B: DNA (100 $\mu\text{g/ml}$) treated with dornase alfa (2.9 $\mu\text{g/ml}$). C: DNA (100 $\mu\text{g/ml}$) treated with dornase alfa (2.9 $\mu\text{g/ml}$) and UFH (1 $\mu\text{g/ml}$). D: DNA (100 $\mu\text{g/ml}$) treated with dornase alfa (2.9 $\mu\text{g/ml}$) and UFH (10 $\mu\text{g/ml}$). E: DNA (100 $\mu\text{g/ml}$) treated with dornase alfa (2.9 $\mu\text{g/ml}$) and UFH (100 $\mu\text{g/ml}$). F: DNA (100 $\mu\text{g/ml}$) treated with UFH (100 $\mu\text{g/ml}$).

through sputum from patients not on dornase alfa therapy. The addition of 0.01 mg/ml UFH caused a nonsignificant increase in nanosphere transport to $17.3 \pm 3.2\%$, while UFH at a concentration of 0.1 mg/ml significantly ($P < 0.05$) increased transport to $25.5 \pm 3.8\%$ of the PBS control compared with untreated CF sputum. UFH was therefore more potent in the presence of dornase alfa, and a concentration of 0.1 mg/ml caused a significant increase in the transport of nanospheres through dornase alfa-treated CF sputum, whereas a higher concentration of 1 mg/ml was required to cause a significant increase in the transport of nanospheres through non-dornase alfa-treated CF sputum. However, the effect of concentrations of UFH >0.1 mg/ml was lost in the samples from dornase alfa-treated patients.

DISCUSSION

Our results confirm the contribution made by DNA to the rheological properties of purulent sputum. This study has also revealed previously unrecognized mucoactive properties of

UFH. UFH dissociates the actin-DNA bundles in CF sputum, reducing the elasticity and yield stress in the samples and enhancing the diffusion of 200-nm nanospheres through sputum. In addition, UFH enhances the endonuclease activity of therapeutic concentrations of dornase alfa. These findings suggest novel heparin-based approaches to improve airway clearance and drug delivery in the CF airway.

The reason(s) for the excessive inflammatory response in CF is unknown, but it is unlikely to be due to a single factor. It may be due to decreased levels of endogenous anti-inflammatory mediators such as IL-10 (13), lipoxin (24), or the collectins surfactant protein (SP)-A and SP-D (38) or increased production of the proinflammatory neutrophil chemoattractant IL-8 (35) or its accessory molecule HSPG (57) in bronchial tissue. Whatever the cause, the inflammatory response overwhelms defective apoptotic and phagocytic mechanisms (1, 61) that normally remove effete neutrophils. Consequently, necrotic neutrophils deliver high concentrations of DNA and actin into the airway secretions, contributing to their abnormal physical properties.

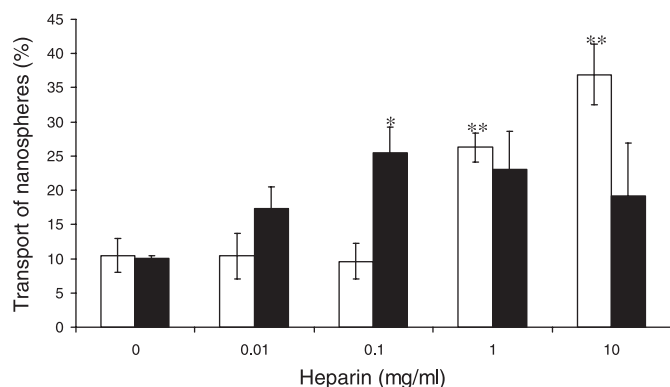


Fig. 8. Effect of UFH on barrier function of sputum from non-dornase alfa-treated (open bars, $n = 4$) and dornase alfa-treated (black bars, $n = 3$) CF patients. Homogenized sputum was treated with UFH (0, 0.01, 0.1, 1, or 10 mg/ml) and mixed with an equal volume of fluorescent 200-nm nanospheres. Treated sputum (20 μ l) was added to the upper well of a micro-Boyden chamber and incubated for 4 h at 37°C. Fluorescence of solution in the lower wells was measured on a plate reader (excitation 485 nm, emission 535 nm). Transport of nanospheres is expressed as a percentage of transport through PBS buffer alone (100%). Results are expressed as means \pm SE. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the absence of UFH.

There are a number of reasons why it would be useful to remove DNA from airway secretions. It not only contributes to the abnormal viscoelasticity of CF sputum (52, 53) and is associated with changes in pulmonary function and sputum cough transportability (26), but it supports *Pseudomonas aeruginosa* biofilm formation (65, 67) and inhibits the activity of cathelicidins (66) as well as aminoglycoside antibiotics (22), while bacterial DNA stimulates the immune system (34).

Our CLSM studies, together with DNA analysis by agarose gel electrophoresis, indicate that heparin disaggregates DNA-actin bundles, releasing intact high-molecular-weight DNA that could not be resolved on CLSM analysis and had apparently disappeared but could be detected on agarose gels. Actin-DNA bundles are stabilized around cationic proteins such as histones, which are released as chromatin is released from necrotic cells, and lysozyme (59). These polyvalent counterions serve to reduce electrostatic repulsion between the anionic polymers and are dissociated by polyvalent co-ions such as poly-aspartate and polyglutamate (59). The mechanism by which polyanionic UFH dissociates DNA-actin bundles may be similar. However, in sputum samples from patients who were receiving dornase alfa therapy, UFH also dose-dependently enhanced the activity of dornase alfa, leading to the complete dissolution of DNA in CF sputum. The enhancement of endonuclease activity is most likely via the ability of UFH to liberate DNA from DNA-actin bundles in CF sputum. However, a direct effect of UFH on dornase alfa, a heparin-binding protein (11), activity has not been ruled out. A direct effect of UFH on bovine pancreatic DNase I activity was indicated using AFM to image networks of calf thymus DNA in the absence of actin. Degradation of the networks by DNase alone, at a concentration achieved clinically (55), was negligible. However, the DNA networks completely dissolved in the presence of low concentrations of both UFH and DNase.

Our results also demonstrate that in vitro treatment with UFH alone reduced the elasticity, without an effect on the viscosity, and increased tan delta (the ratio of viscosity to elasticity) of sputum samples from patients not receiving dor-

nase alfa therapy to levels that were not different from those of patients who were receiving dornase alfa therapy. This effect on sputum rheology appears to be mediated by disaggregation of the DNA-actin bundles, since more intact DNA was recovered from the samples. Both elasticity and tan delta are important determinants of mucus transportability (28), which suggests that inhaled UFH alone could improve mucus clearance. For both elasticity and tan delta measurements, there were no significant differences for the values obtained at 1 and 10 mg/ml UFH, indicating that 1 mg/ml may be an optimum concentration.

The effect of UFH on sputum rheology was restricted to the group of patients not receiving dornase alfa therapy, presumably because dornase alfa (54) and UFH (this study) both target the DNA-actin bundles that contribute to sputum elasticity (60). UFH had no effect on the residual elasticity in sputum from dornase alfa-treated patients; this is believed to be due predominantly to the mucin network with a contribution from actin polymers (36). Previous studies of the mucolytic effects of polysaccharides indicated that the uncharged low-molecular-weight dextran polysaccharides thin mucus by breaking hydrogen bonds in the mucin glycoprotein network (30). The charged polysaccharides, dextran sulfate, and low-molecular-weight heparin were more effective mucolytics; this was suggested to be due to a combination of hydrogen bond breaking in the mucin network, interaction with positive regions of the mucin molecule, and charge shielding by mobile counterions (30). Ours is the first report that UFH has effects on the DNA-actin bundles in CF sputum, in addition to the previously reported effects of low-molecular-weight heparin on mucin networks.

DNA in CF sputum has been reported to be a significant barrier to the delivery of gene therapy vectors (58). Although UFH was more potent in the presence of dornase alfa, the ability of UFH alone to enhance the diffusion of 200-nm nanospheres through CF sputum suggests a new heparin-based approach to the challenge of improving access of vectors to target cells. High elasticity arrests the transport of particles across CF sputa (18), and a decrease in the elasticity of sputum is expected to increase diffusion. Thus heparin may increase particle diffusion by decreasing polymer interactions and elasticity. However, diffusion of particles is through fluid-filled pores in the polymer network, and the microviscosity within the pores is lower than the macroviscosity (18). It was suggested that, on treatment with dornase alfa, DNA fragments fill the pores in the polymer network, increasing the microviscosity and ultimately limiting diffusion through the pores (18). This may explain the bell-shaped curve seen in Fig. 8 for the effect of heparin on particle diffusion through samples from dornase alfa-treated patients.

Ledson et al. (31) reported that, in adult CF patients, inhalation of 25,000 IU of heparin daily for 7 days was safe, and patients found sputum easier to expectorate. We recently reported the safety of inhaled heparin at 50,000 IU twice daily for 14 days in adult CF patients, although we were unable to demonstrate a statistically significant effect on mucus rheology at the doses used (51). However, since only ~8% of inhaled nebulized UFH reaches the lower respiratory tract (2), and it has been estimated that there are up to 150 ml of secretions in the CF airway (4), an effective dose of 1 mg/ml (168 U/mg in this study) would require inhalation from solutions containing

up to 315,000 units. Inhaled UFH does not cross the bronchial mucosa to induce clinically significant changes in coagulation parameters even at very high doses, up to 400,000 IU, in healthy subjects (3). However, further clinical studies at higher doses are needed to investigate the safety and efficacy of UFH compared with other mucolytic agents, such as hypertonic saline and mannitol, which are in development for the treatment of CF lung disease.

Our data indicate that UFH alone has a number of beneficial effects on CF sputum; however, a combination of UFH and dornase alfa cleaves DNA more effectively than dornase alfa alone and could therefore be more effective clinically. The use of UFH would also overcome concerns that dissolution of DNA releases bound proinflammatory mediators such as IL-8 (41) and neutrophil elastase (64) into the airways, since heparin is an effective inhibitor of the function of IL-8 (43) and neutrophil elastase activity (23).

In summary, we have demonstrated novel mucoactive properties of UFH that, together with its well-documented anti-inflammatory properties, support a role for inhaled heparin in the treatment of CF lung disease.

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Some of the results of this study have been published previously in abstract form (7).

GRANTS

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