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Reduced Three-Dimensional Motility in Dehydrated Airway Mucus Prevents Neutrophil Capture and Killing Bacteria on Airway Epithelial Surfaces¹

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Cystic fibrosis (CF) lung disease is characterized by persistent lung infection. Thickened (concentrated) mucus in the CF lung impairs airway mucus clearance, which initiates bacterial infection. However, airways have other mechanisms to prevent bacterial infection, including neutrophil-mediated killing. Therefore, we examined whether neutrophil motility and bacterial capture and killing functions are impaired in thickened mucus. Mucus of three concentrations, representative of the range of normal (1.5 and 2.5% dry weight) and CF-like thickened (6.5%) mucus, was obtained from well-differentiated human bronchial epithelial cultures and prepared for three-dimensional studies of neutrophil migration. Neutrophil chemotaxis in the direction of gravity was optimal in 1.5% mucus, whereas 2.5% mucus best supported neutrophil chemotaxis against gravity. Lateral chemokinetic movement was fastest on airway epithelial surfaces covered with 1.5% mucus. In contrast, neutrophils exhibited little motility in any direction in thickened (6.5%) mucus. In *in vivo* models of airway mucus plugs, neutrophil migration was inhibited by thickened mucus (CF model) but not by normal concentrations of mucus ("normal" model). Paralleling the decreased neutrophil motility in thickened mucus, bacterial capture and killing capacity were decreased in CF-like thickened mucus. Similar results with each mucus concentration were obtained with mucus from CF cultures, indicating that inhibition of neutrophil functions was mucus concentration dependent not CF source dependent. We conclude that concentrated ("thick") mucus inhibits neutrophil migration and killing and is a key component in the failure of defense against chronic airways infection in CF. *The Journal of Immunology*, 2005, 175: 1090–1099.

Chronic bacterial infection of the airways is the major cause of morbidity and mortality in cystic fibrosis (CF)³ patients (1). Despite advances in CF research, the pathogenesis of CF lung disease is not completely understood. In general, it appears that CF airways disease reflects a reduction in airway surface liquid (ASL) volume in both the mucus and periciliary liquid layers (2). ASL depletion produces thickened (concentrated) mucus gels that adhere to airway surfaces (3, 4) and become the site for the chronic intraluminal infection characteristic of CF lung disease (5). However, other major lung defense systems as well may not function properly in CF airways covered with concentrated mucus. Elucidation of dysfunction of these systems in the context of the reduced ASL volume characteristic of CF may help explain the complexity and severity of CF lung disease.

In airway defense, mucus clearance is supplemented by airway macrophages that patrol airway surfaces and eradicate inhaled bacteria in concert with antimicrobial proteins (6) and other proteins such as IgA and collectins (7). When these surface defense pro-

cesses cannot contain an inhaled bacterial load, neutrophils are recruited from the blood to kill bacteria. As compared with alveolar pneumonias, neutrophils in airway infections have to penetrate mucus and move in three dimensions to capture and kill bacteria. However, there have been no investigations of neutrophil function in the three-dimensional environment that characterizes mucus-covered airway surfaces. Instead, studies of neutrophil migration in the airways have focused on how neutrophils migrate from the capillaries through the extracellular matrix and onto the epithelial surface, not how they move through the actual infection site, *i.e.*, mucus (8, 9).

Accordingly, we designed experimental systems to investigate how neutrophils penetrate mucus to capture bacteria and whether these processes are affected by the abnormally thickened (concentrated) mucus that characterizes the CF airway. First, we examined two basic features of neutrophil motility in mucus of varying concentrations and containing a chemoattractant, *i.e.*, chemotaxis and chemokinesis. The conventional devices to measure neutrophil migration in gels have measured migration in either the downward (with gravity) or lateral directions (10–13). However, because 50% of airway surfaces face the "upward direction" (antigravitational) *in vivo*, we designed assays to measure both upward and downward neutrophil migration through mucus. These *in vitro* studies were complemented by *in vivo* studies in xenografts. Second, we measured the capacity of neutrophils to capture and kill bacteria in normal and thickened (concentrated) mucus using heat-killed and live bacteria. Finally, to mimic the *in vivo* situation, studies of neutrophil-bacteria interactions over a range of mucus concentrations were performed with both ciliated and nonciliated human airway epithelial cultures. For all studies, mucus was obtained from human airway epithelial cultures and processed to generate mucus concentrations that spanned normal and CF values.

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³ Abbreviations used in this paper: CF, cystic fibrosis; ASL, airway surface liquid; HBE, human bronchial epithelial; WGA-Rh, wheat germ agglutinin conjugated to tetramethylrhodamine.

Materials and Methods

Human bronchial epithelial (HBE) cell isolation and culture

HBE cells were isolated and cultured under air-liquid conditions as previously described, with certain modifications (14). The modifications required to collect mucus included plating 1×10^6 passage-2 HBE cells on collagen-coated permeable supports (Transwell-Col, 24 mm in diameter, 0.4- μ m pore; Costar). To prepare ciliated and nonciliated cultures for neutrophil migration studies, passage-2 HBE cells were seeded on Transwell-Col (12 mm). Day 5–14 cultures were nonciliated, and day 21–60 cultures were ciliated, as defined by the presence or absence of beating cilia with microscopy (TE300; Nikon).

Preparation of concentrated mucus

After HBE cells started to produce mucus, 200 μ l of PBS were added to the apical surface, and 24 h later, all liquid (mucus) was aspirated. The material harvested from cultures was pooled and stored at 4°C. The mucus samples were placed in dialysis tubes (MWCO = 3500; Spectrum Laboratories) and concentrated with a polymer absorbent (Spectra/Gel; Spectrum Laboratories) for 1–5 days at 4°C until the mucus reached the target volume, which was calculated from the percent dry weight and the volume of the initial mucus sample. To generate an isotonic sample, the concentrated mucus was dialyzed against PBS (2 liters \times 2) containing 500 μ M MgCl₂ and 800 μ M CaCl₂ at 4°C, then 1/100 volume of D-glucose solution (100 mg/ml) were added (final concentration, 1 mg/ml). The percent dry weight was calculated by measuring wet and dry weights with a microbalance (model 27; Cahn Electronic Balance) before and after drying the sample at 80°C overnight (15). Because of the difficulty in depleting harvested mucus samples of exact volumes, a 10% difference from the target concentration was allowed.

Determination of mucin content of mucus

The concentrations of mucins in HBE cell culture secretions were assessed by gel chromatography and differential refractometry (dn/dc). A value for dn/dc of 0.165 ml/g was used for mucins, which we have measured previously at 650 nm. To measure mucin concentrations, samples were diluted in running buffer in the range 1/20 to 1/40 to maintain a response within the dynamic range of the instrument, and a 500- μ l sample was chromatographed on a column of bed volume (12 ml) containing Sepharose CL-2B (Amersham Biosciences). The column was eluted with 200 mM sodium chloride/10 mM EDTA at a flow rate 0.3 ml/min. The column effluent was passed through an in-line Dawn enhanced optimal system laser photometer coupled to a Wyatt/Optilab digital signal processing interferometric refractometer to measure light scattering and sample concentration, respectively. Light scattering and refractive index measurements were made continuously, with the light scattering being used only to identify and define the mucin peak. The captured data were integrated and analyzed with the Astra software provided with the Dawn. Optimum dilutions were sought by trial and error and then paired values were obtained to check reproducibility, which was typically within 5%.

Characterization of the structure and function of mucus samples

The topography of mucus samples of different concentrations was visualized with wheat germ agglutinin (lectin from *Triticum vulgaris*) conjugated to tetramethylrhodamine (WGA-Rh; Molecular Probes). A 100- μ l sample of each mucus concentration was placed on a coverslip chamber (Cover-Well 0.5 \times 20 mm; Grace Biolaboratories) with 1 μ l of 5 mg/ml WGA-Rh and visualized by confocal microscopy (Zeiss Laser Scanning Microscope 510; Carl Zeiss) with a \times 40 objective (numerical aperture, 1.3; oil immersion). Pore diameters in the mucus meshwork were estimated with a Metamorph image analysis system (Universal Imaging) by tracing the mucin fibers surrounding the pores (100 pores/image from three images per concentration) and calculating cross-sectional areas. Equivalent pore diameters were calculated as the diameters of perfect circles having the same areas.

Mucus transport was measured for the different percentage of solids samples on well-differentiated, fully ciliated epithelial cultures. As reported previously (2, 14), mucus is transported in a rotational fashion on the surfaces of cultures when mucus is physiologically hydrated, whereas concentrated mucus is stationary. Each mucus sample (30 μ l) was mixed with 1 μ l of a 0.2% suspension of fluorescent microspheres (yellow-green, carboxylate-modified, 1 μ m in diameter; Molecular Probes) and placed on well-differentiated HBE cultures. Pictures were taken with an epifluorescent microscope (Eclipse TE300; Nikon) equipped with a digital camera (DVC1312M; DVC) with a \times 4 objective and a 5-s exposure.

Neutrophil chemotaxis in mucus

Neutrophils were isolated from normal healthy adult volunteers, as reported previously (16) (protocols approved by the University of North Carolina Institutional Review Board). Neutrophils, suspended in HBSS containing 500 μ M MgCl₂ and 800 μ M CaCl₂, were labeled with calcein-AM (10 μ M) for 30 min at 37°C.

For the gravitational (“downward”) chemotaxis measurements (see Fig. 2*Ai*), 10 μ l of 100 nM fMLP (Sigma-Aldrich) were added to the bottom of 24-well culture plates and allowed to dry. Then, 200 μ l of mucus (1.5, 2.5, or 6.5% dry weight) were added, and a Transwell (6.5 mm in diameter, 3.0- μ m pore, polycarbonate; Costar) placed atop the mucus (Fig. 2*Ai*). A neutrophil suspension in mucus was prepared by adding 1.5×10^3 calcein-labeled neutrophils to 30 μ l of mucus and added to the Transwell lumen. After 3-h incubation, the number of neutrophils that had migrated through the Transwell membrane into the lower compartment was quantitated with a fluorescent microscope (Eclipse TE300; Nikon).

For antigravitational (“upward”) chemotaxis measurements (Fig. 2*Bi*), 400 μ l of a neutrophil suspension (5×10^4 cells/ml) were added to each well of 24-well culture plates and incubated for 30 min at 37°C to allow neutrophils to sediment to the bottom of the well. After aspiration of the supernatant, 200 μ l of mucus (1.5, 2.5, or 6.5%) were added, and a Transwell culture insert (6.5 mm in diameter, 3.0- μ m pore, polycarbonate; Costar) placed on the mucus, following which a filter paper containing 10 μ l of 100 nM fMLP was placed on the inner surface of the Transwell membrane. After a 3-h incubation at 37°C, the number of neutrophils migrating $>100 \mu$ m from the bottom of the well into mucus was counted with an epifluorescence microscope.

Neutrophil chemokinesis

After migrating to the airway surface from the submucosa, neutrophils contact the mucus covering the epithelial surface. Neutrophils can either migrate into mucus or move along the epithelial surface. Lateral motility (epithelial surface movement) of neutrophils was measured on nonciliated airway epithelial cultures by depositing a calcein-labeled neutrophil suspension (2×10^4 cells in 200 μ l) on nonciliated airway epithelial culture surfaces for 30 min to allow neutrophils to sediment. The supernatant was gently aspirated, mucus samples (1.5, 2.5, and 6.5%) containing 10 nM fMLP added to the epithelial surfaces, and the preparations placed in a heated chamber (δ TC3; Biopetechs) on an epifluorescent microscope. Movement of neutrophils was recorded at 1-min intervals for 15 min at 37°C, and the tracks analyzed with a Metamorph image analysis system (Universal Imaging). Four series of images with >10 neutrophils each were obtained and analyzed for each mucus concentration.

Neutrophil migration into normal and CF xenografts

Tracheal xenografts were created as previously described (17), with the following modifications. Briefly, rat tracheas were denuded of their own epithelium and both ends cannulated with polyethylene tubing. Between 0.5 and 1 million passage 1 or 2 human CF or non-CF airway epithelial cells were placed into the lumen, cannulas ligated with vascular clips, grafts implanted s.c. into the interscapular space of Nu/Nu mice, and allowed to mature for 14–19 days. The mice were then anesthetized, graft ends revealed surgically, ligatures removed, and the lumen injected with varying concentrations of mucus containing 100 nM fMLP. Xenograft model tracheas seeded with non-CF epithelia were injected with 2.5% mucus as a normal airway model, whereas tracheas seeded with CF epithelia were injected with 8% mucus as a CF airway model. After 6 h, grafts were removed and rapidly frozen for sectioning. Neutrophil number was counted with image analysis software after acquiring digital images of five sections for each xenograft. Xenograft sections with unepithelialized surfaces or inhomogeneous mucus distribution were excluded from analysis. The number of neutrophils was counted in the entire lumen and the outer half to calculate the peripheral to central ratio of neutrophil distribution. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Preparation of bacteria

Heat-killed, fluorescently labeled *Escherichia coli* (K-12 strain, tetramethylrhodamine conjugate; Molecular Probes), preopsonized for 30 min with rabbit polyclonal Abs (Molecular Probes), was used to examine the capability of neutrophils to capture immotile (dead) bacteria. *Pseudomonas aeruginosa* was used to examine neutrophils for their ability to capture motile “live” bacteria. An overnight culture of *P. aeruginosa* PAO1 (18), grown on trypticase soy agar (Oxoid), was prepared for each assay. In addition, we also used a PAO1 strain carrying plasmid pTdk-*gfp* (19) (GFP-PAO1). This strain constitutively expresses the stable form of GFP.

To maintain the plasmid, cultures were grown for each assay overnight on trypticase soy agar in the presence of carbenicillin (200 $\mu\text{g/ml}$).

Neutrophil capture of heat-killed bacteria in mucus on ciliated and nonciliated HBE cultures

To test whether neutrophil motility (chemotaxis and chemokinesis) in mucus of varying concentrations affected bacterial capture, we incubated calcein-labeled neutrophils with preopsonized, heat-killed *E. coli* (tetramethylrhodamine-labeled). Two hundred microliters of a neutrophil suspension (2×10^4 cells) were added to the apical surface of nonciliated HBE cultures. After the neutrophils had sedimented onto the surface, the supernatant was gently aspirated, and bacteria suspended in mucus (2×10^5 bacteria/50 μl of mucus) were placed on the epithelial surface. After overnight incubation, pictures were taken with epifluorescence and confocal microscopes (*x-z* scanning). The number of neutrophil-associated bacteria was counted with an epifluorescent microscope. For *x-z* confocal scanning, epithelial cells were counterstained with 5 μM calcein-AM.

Because preliminary experiments revealed that beating cilia prevented neutrophil sedimentation onto the surface of ciliated cultures, neutrophils (2×10^4) and bacteria (2×10^5) were mixed and suspended in 50 μl of mucus and placed on ciliated cell cultures. After overnight incubation, the number of neutrophil-associated bacteria was counted with an epifluorescent microscope. The percent dry weight of the mucus was not changed significantly during the incubation (data not shown).

*Neutrophil capture of live *P. aeruginosa* in mucus*

We used GFP-PAO1 to measure capture of live bacteria by neutrophils in mucus. Two hundred microliters of a neutrophil suspension (5×10^5 cells/ml) were placed in a plastic coverslip chamber (0.5×20 mm; Grace Biolaboratories). After neutrophils had sedimented onto the plastic surface (~ 30 min), the supernatant was aspirated, and 100 μl of mucus containing 1×10^6 GFP-PAO1 (bacteria:neutrophils = 10:1) were added to the chamber. After a 2-h incubation at 37°C, the number of neutrophil-associated bacteria was counted with epifluorescence microscopy. For this experiment only, neutrophils were labeled with a red fluorophor (5-(and-6)-chloromethyl SNARF-1; Molecular Probes).

*Neutrophil killing of *P. aeruginosa* in mucus on ciliated and nonciliated airway cultures*

For all experiments with live bacteria, cell cultures were washed with PBS 24 h before the experiment and incubated with cell culture medium in the antibiotic-free basolateral bath. For nonciliated cell cultures, 200 μl of a neutrophil suspension ($1 \times 10^5/\text{ml}$) were added to the apical culture surface. After neutrophils sedimented onto the surface, the supernatant was aspirated, and bacteria suspended in mucus (500 bacteria/50 μl of mucus) were added. The ratio of neutrophils to bacteria was $\sim 40:1$. In preliminary experiments, ratios $> 10:1$ exhibited significant bacterial killing. After a 2-h incubation at 37°C, the number of live bacteria was determined by serial dilution and plating on sheep blood agar plates (BD Biosciences). The ratio of bacterial number 2 h after plating, with vs without neutrophils, was used to express bacterial killing mediated by neutrophils.

When ciliated cultures were used, neutrophils (2×10^4), bacteria (500), and mucus (50 μl) were mixed before addition to the cell cultures. The sample was then placed on ciliated bronchial epithelial cultures. Control experiments were performed with a mucus-bacterial mixture without the addition of neutrophils. After a 2-h incubation at 37°C, the mucus was harvested with PBS containing 10 mM DTT (20), the number of live bacteria determined by serial dilution and plating on 5% blood agar plates, and the percent bacterial number used to measure neutrophil effect, as above.

Characterization of mucin species by HPLC ion-exchange chromatography

Mucus samples were dialyzed into urea reduction buffer (6 M urea containing 0.1 M Tris/5 mM EDTA (pH 8.0)) and treated with 10 mM DTT for 2 h at 37°C. Iodoacetamide was added to a final concentration of 25 mM, and the mixture was left in the dark for 30 min at room temperature. The reduced samples were chromatographed on an Amersham Biosciences MonoQ HR 5/5 column, using Amersham Biosciences, Ettan LC high-pressure chromatography system. The column was eluted with a linear gradient of 0–0.75 M lithium perchlorate/10 mM piperazine in 6 M urea (pH 5.0) with a flow rate 1 ml/min, and 0.5-ml fractions were collected. Aliquots from the fractions were transferred to the nitrocellulose membrane by slot blotting using a Schleicher and Schuell blotter. Mucins were detected on the membrane by standard chemiluminescence assay, using the polyclonal antisera (MAN-5AC1, MAN-5B1) to MUC5AC and MUC5B mucins, respectively. The relative intensity of the slot blot bands was mea-

sured by densitometry and quantitated by ImageQuaNT (Molecular Dynamics) software.

Characterization of CF vs normal mucin species by western blotting after agarose gel electrophoresis

Mucins may be dispersed in size and charge density due to differential glycosylation. Thus, electrophoretic behavior cannot be matched simply to m.w. and/or size. However, combined with ion-exchange chromatography, it does yield a qualitative idea relating mucin size distribution to charge density, i.e., the homogeneity or heterogeneity of the preparation. This is how the technique was used here. Mucins fractionated by ion-exchange chromatography were electrophoresed in 1.0% (w/v) agarose gel (15×15 cm) in 40 mM Tris-acetate/1 mM EDTA (pH 8.0) containing 0.1% (w/v) SDS. Electrophoresis was performed in a horizontal gel apparatus using Bio-Rad SubCell GT (75 ml of gel, 1500 ml of buffer, 82 V, and 2.5 h at room temperature). After electrophoresis, subunits were transferred to nitrocellulose by vacuum blotting in 0.6 M sodium chloride/0.06 M sodium citrate, using an Amersham Biosciences VacuGene XL at a suction pressure of 45 mbar for 1 h before detection of mucins (21). Mucins were detected by using polyclonal antisera MAN-5AC1 and MAN-5B1 raised against MUC5AC and MUC5B mucins, respectively (22).

Neutrophil functions in CF airway mucus

Mucus from CF airway epithelial cultures was collected, concentrated, and dialyzed as stated above, except 300–500 μl of PBS were needed to collect mucus due to hyperabsorption by CF airway epithelia over the 24-h period. Three experiments, gravitational and antigravitational neutrophil chemotaxis and bacterial killing by neutrophils on ciliated surface, were repeated using CF mucus with varying concentrations (1.5, 2.5, and 6.5% solids) to test whether neutrophil functions are dependent on mucus concentration or on CF or non-CF origin.

Statistics

Unless otherwise stated, all data are expressed as the mean \pm SE. Comparisons among mucus samples of three concentrations were made with ANOVA and Tukey test. Comparisons were made with Student's *t* test between normal and CF-like mucus samples with $p < 0.05$ considered significant.

Results

Characterization of mucus samples

To determine the biochemical composition of mucus samples of varying concentrations, total mucins within mucus samples of varying concentrations were measured. In all the mucus samples, mucins constituted $\sim 60\%$ of the nonionic components (Fig. 1A). As mucus was concentrated from 1.5 to 6.5% solids, mucin concentration increased > 10 times from 2.64 to 31.2 mg/ml. Given the presence of 1% salt in isotonic mucus samples, the mucin concentration should theoretically increase 11 times over this concentration range.

The gel-forming character of mucus derives mainly from properties of mucin MUC5AC and MUC5B macromolecules, which form a mesh-like entangled network (23, 24). To ascertain whether mucus maintained its network structure after manipulation (pipetting, dehydration, dialysis), we stained mucus of three different concentrations with WGA-Rh (Fig. 1B). The network was inhomogeneous in 1.5% mucus, with large holes and variability of the mesh size (pore diameters ranged between 1.5 and 40.1 μm ; 95% range, 7.6 ± 8.6 μm ; mean \pm SD). A regular mesh-like network of fibers was detected in 2.5% mucus; pore diameters ranged between 1.6 and 14.1 μm (95% range, 5.3 ± 3.8 μm ; mean \pm SD). In contrast, mesh-like pores in 6.5% mucus were too small to resolve.

We also measured the transport of mucus on ciliated cultures as a functional characterization of these mucus preparations. Microspheres embedded in 1.5% mucus were transported in a complex fashion, with variations in bead vector and velocity (Fig. 1Ci). Fluorescent microspheres trapped in 2.5% mucus exhibited organized rotational movement, with a velocity similar to that reported

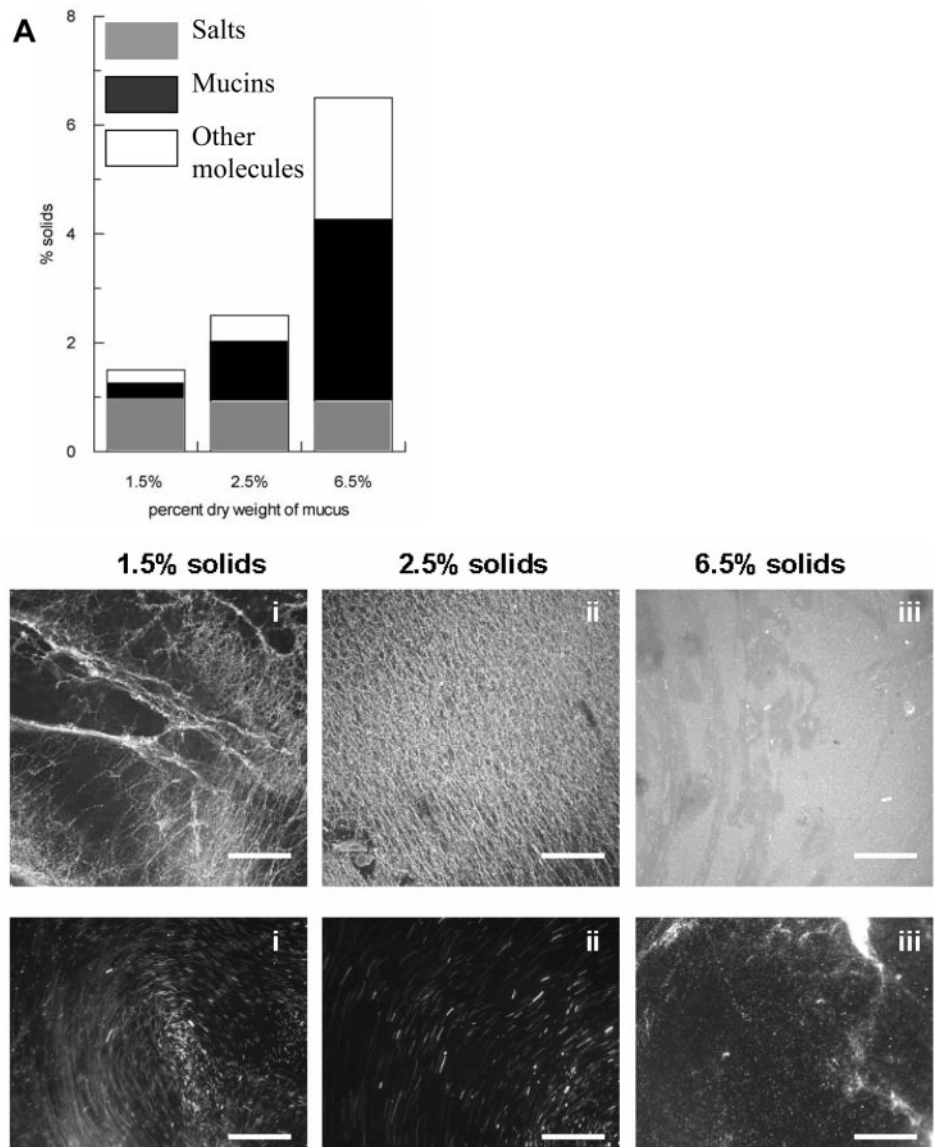


FIGURE 1. Characterization of normal and CF-like mucus samples of different concentrations. *A*, Compositions of mucus samples. Total mucin content was measured with a differential refractometer. *B*, Topography of mucus samples of different concentrations. Samples were stained with WGA-Rh and photographed with a $\times 40$ objective by *x-y* confocal microscopy: *i*, 1.5% mucus; *ii*, 2.5% mucus; and *iii*, 6.5% mucus. Scale bars = 50 μm . *C*, Epithelial transport of three mucus samples. Mucus was labeled with 1.0- μm fluorescent microspheres and placed on a ciliated epithelial cell culture and rotational transport photographed with a $\times 4$ objective and 5-s exposure. The lines are the tracks of fluorescent microspheres. *i*, 1.5% mucus; *ii*, 2.5% mucus; and *iii*, 6.5% mucus. Scale bars = 0.5 mm.

in vivo (14) (Fig. 1*Cii*). In 6.5% mucus, microspheres were poorly transported (Fig. 1*Ciii*), consistent with the concept that 6.5% solids mucus is abnormally concentrated.

Neutrophil chemotaxis

Neutrophil chemotaxis in the direction of gravity was measured between two compartments separated by a 10- μm -thick polycarbonate membrane with 3.0- μm pores. The number of neutrophils migrating to the lower compartment was greatest in 1.5% mucus (28.3 ± 5.2 cells/well), smaller in 2.5% mucus (0.4 ± 0.2 cells/well), and absent in 6.5% mucus (0 ± 0 cells/well) (Fig. 2*Aii*) (ANOVA, $p < 0.0001$ and Tukey test, $p < 0.01$ for both 1.5 vs 2.5 and 1.5 vs 6.5%).

To test whether neutrophils could migrate against gravity in mucus, neutrophil chemotaxis was measured in a chamber with mucus separating neutrophils (bottom) from a fMLP source (Fig. 2*Bi*, top). In contrast with the experiments in the direction of gravity, only 2.5% mucus supported neutrophil movement against gravity (Fig. 2*Bii*) (ANOVA, $p < 0.0001$ and Tukey test, $p < 0.01$ for both 1.5 vs 2.5 and 2.5 vs 6.5%).

Neutrophil chemokinesis

To measure the lateral motility of neutrophils on nonciliated epithelial surfaces covered with mucus of varying concentrations, neutrophils were tracked at 1-min intervals for 15 min (Fig. 3*A*) and mean velocities calculated (Fig. 3*B*). Chemokinetic motility was significantly lower on surfaces covered by 6.5% mucus than 1.5 or 2.5% mucus (Fig. 3*B*). Because neutrophils could migrate in the *z*-axis in 2.5% mucus (Fig. 2*Bii*), the mean velocity of neutrophils tracked in the two-dimensional images was likely underestimated.

Neutrophil migration into mucus within normal and CF xenografts

Neutrophil motility was then examined in an in vivo airway model, i.e., the tracheal xenograft that incorporates neutrophil migration from the vascular compartment, through the epithelium, and into an airway lumen filled with mucus. Neutrophils migrated into tracheal xenografts (both normal and CF models) filled with mucus containing fMLP after a 6-h incubation. The neutrophil distribution was homogeneous in normal xenografts (periphery:center

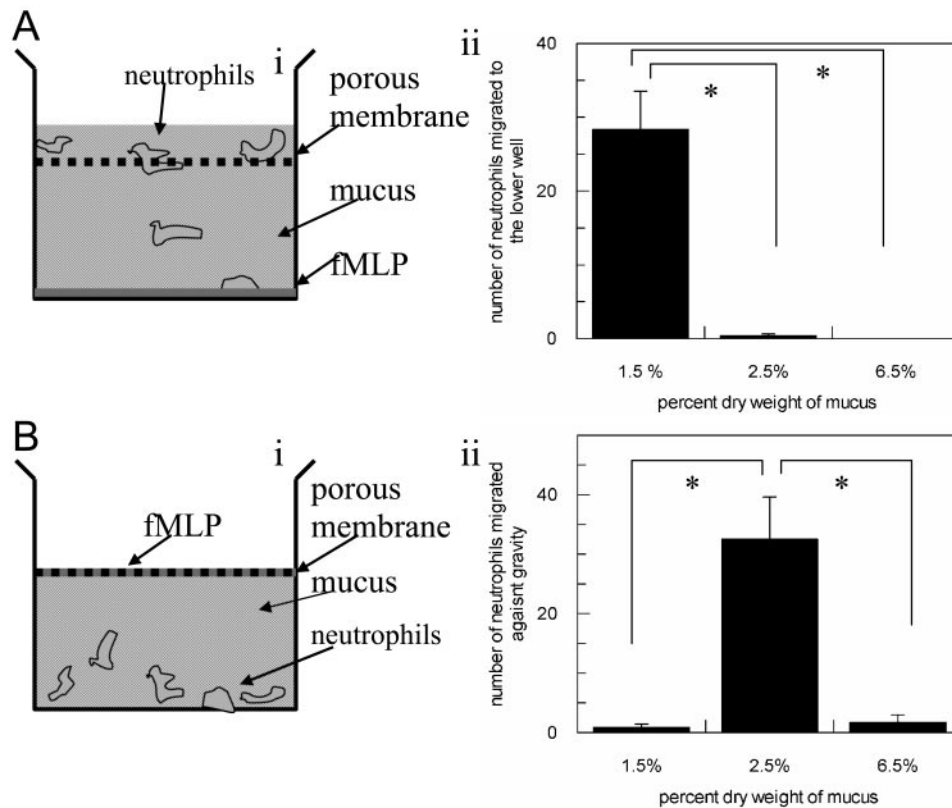


FIGURE 2. Neutrophil chemotaxis in the gravitational and antigravitational directions in mucus. *Ai*, Diagram of chemotaxis apparatus to measure chemotaxis in the direction of gravity. fMLP was added to the bottom of a well and allowed to air dry. Mucus (1.5, 2.5, or 6.5% percent dry weight) was then added to this compartment. A suspension of fluorescently labeled neutrophils in mucus was added to a Transwell insert (6.5 mm in diameter, 3.0-mm pore, polycarbonate) and placed on the mucus. *Aii*, Mean neutrophil migration in the direction of gravity. After 3-h incubation, the number of neutrophils that migrated through the Transwell membrane was counted in the lower compartment (mean \pm SE, $n = 6$; *, $p < 0.05$). *Bi*, Diagram of chemotaxis apparatus to measure antigravitational chemotaxis. Fluorescently labeled neutrophils were sedimented onto the bottom of a well, and mucus was added onto them. A filter paper containing 10 μ l of 100 nM fMLP was placed in a 6.5-mm Transwell insert and placed on the surface of the mucus. *Bii*, Mean neutrophil migration against gravity (mean \pm SE, $n = 6$; *, $p < 0.05$).

ratio ≈ 1 ; Fig. 4, *A* and *C*). In contrast, neutrophil migration into mucus was slowed at the periphery of the lumen in CF xenografts (Fig. 4*B*), with significantly fewer neutrophils reaching to the center of the mucus plug (Fig. 4*C*). Thus, it appears that concentrated mucus presents a significant barrier to neutrophil migration in vivo.

Capture of heat-killed bacteria in mucus on ciliated and nonciliated epithelial cultures

To examine whether the low motility of neutrophils in concentrated mucus also degraded their capacity to capture bacteria, we first studied neutrophil capture of heat-killed bacteria as a model of immotile bacteria in mucus of varying concentrations. Following overnight incubation with 1.5% mucus (Fig. 5, *Ai* and *Bi*), heat-killed bacteria sedimented from the mucus and both neutrophils, and bacteria were concentrated near the epithelial surface. In contrast, both neutrophils and bacteria were detected throughout the mucus layer when the epithelial surface was covered by 2.5% mucus (Fig. 5, *Aii* and *Bii*). However, in both mucus concentrations, the distribution of neutrophils overlapped that of bacteria. With 6.5% mucus, the neutrophils were predominately located on epithelial cell surfaces, whereas bacteria were distributed throughout the mucus layer (Fig. 5, *Aiii* and *Biii*), i.e., their distributions did not overlap.

The number of bacteria captured by neutrophils was quantitated as the number of red particles in green neutrophils (Fig. 5*C*). Neutrophils on nonciliated epithelial surfaces covered by 1.5% mucus

moved efficiently and captured sedimented bacteria (651.4 ± 21.2 bacteria/100 neutrophils). Neutrophils on epithelial surfaces covered by 2.5% mucus captured an intermediate number of bacteria (38.2 ± 12.2 bacteria/100 neutrophils), reflecting the offsetting variables of decreased bacterial sedimentation onto epithelial surfaces vs the capacity of neutrophils to migrate vertically into mucus. Importantly, neutrophils in 6.5% mucus captured few bacteria (2.0 ± 0.9 bacteria/100 neutrophils) due to impaired neutrophil lateral and vertical motility.

On ciliated epithelia (Fig. 5*D*), the results were similar to those with nonciliated epithelia. The number of bacteria captured by neutrophils was highest in 1.5% mucus (188.3 ± 48.6 bacteria/100 neutrophils), although this number was less than the number of bacterial captured on nonciliated surfaces. The reduced capture on ciliated cultures likely reflected the reduction of sedimentation of bacteria onto cell surfaces due to ciliary beating. The 2.5 and 6.5% values were higher relative to nonciliated cultures, probably due to premixing of neutrophils with bacteria. However, the number of bacteria captured in 2.5% mucus (60.7 ± 4.9 bacteria/100 neutrophils) and 6.5% mucus (21.3 ± 10.3 bacteria/100 neutrophils) was reduced compared with 1.5% mucus.

Capture of *P. aeruginosa* in mucus

We then examined neutrophil capture of live motile bacteria in mucus of varying concentrations, using a PAO1 strain of *P. aeruginosa*. Bacterial velocity was highest in 1.5% mucus and lowest in 6.5% mucus (data not shown). After 2-h incubation of

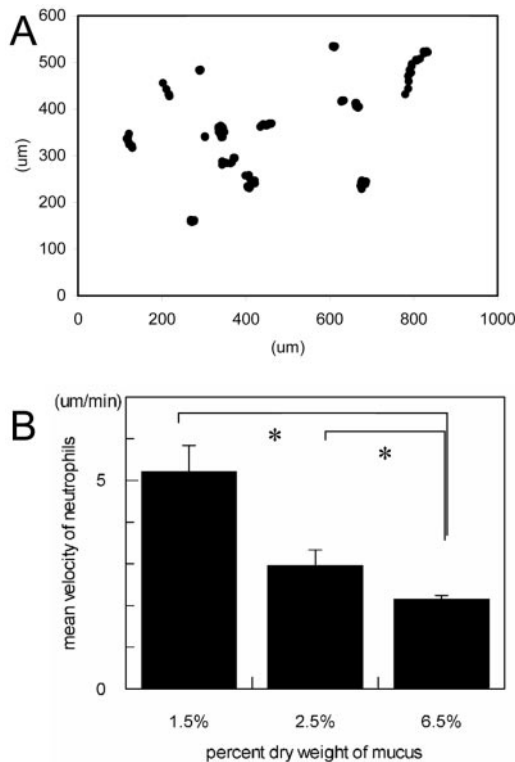


FIGURE 3. Neutrophil chemokinesis on airway epithelial cells covered with the mucus of three concentrations. Lateral motility of neutrophils was measured on nonciliated airway epithelial cultures by depositing a fluorescent-labeled neutrophils on nonciliated airway epithelial cultures. Positions of neutrophils were recorded with epifluorescence microscope at 1-min intervals for 15 min at 37°C, and velocity was calculated. *A*, An example of neutrophil tracks on nonciliated airway epithelia covered with 1.5% mucus showing tracks of 14 neutrophils in a single field of microscopy. Single dots represent no migration, and connected dots represent neutrophil migration during the observation period. *B*, Mean velocity of neutrophils on epithelial surfaces covered by mucus of varying concentrations containing fMLP (mean \pm SE; *, $p < 0.05$).

neutrophils with GFP-PAO1, the number of bacteria associated with bacteria was counted. Neutrophils captured more GFP-PAO1 after a 2-h incubation in 1.5% mucus (120.3 ± 6.5 bacteria/100 neutrophils) than in 2.5% mucus (48.3 ± 3.3 bacteria/100 neutrophils) (Fig. 6A). In 6.5% mucus, the motility of both bacteria and neutrophils was significantly reduced, resulting in only four bacteria being captured by 100 neutrophils.

Killing *P. aeruginosa* in mucus on nonciliated and ciliated airway surfaces

Neutrophil killing of bacteria in mucus was studied in ciliated and nonciliated airway epithelial cultures, mimicking the in vivo conditions of airway bacterial infection. We used a *P. aeruginosa* PAO1 strain to measure bacterial killing activity by neutrophils in 1.5, 2.5, and 6.5% solids mucus, using 500 live bacteria to mimic early infection. On nonciliated epithelia, neutrophils killed bacteria sufficiently well to reduce bacterial number almost in half in 1.5% mucus ($57.1 \pm 2.1\%$ survival) and in 2.5% mucus ($52.6 \pm 4.6\%$ survival). In contrast, neutrophils were completely ineffective in reducing bacterial numbers in 6.5% mucus ($139.4 \pm 29.3\%$ survival) (Fig. 6B). Similar results were obtained on ciliated epithelial cultures (Fig. 6C). The number of bacteria was reduced significantly by incubation with neutrophils in 1.5% mucus ($55.7 \pm 4.7\%$ survival) and in 2.5% mucus ($50.6 \pm 3.5\%$ survival) but not in 6.5% mucus ($103.6 \pm 21.6\%$ survival) (Fig. 6C).

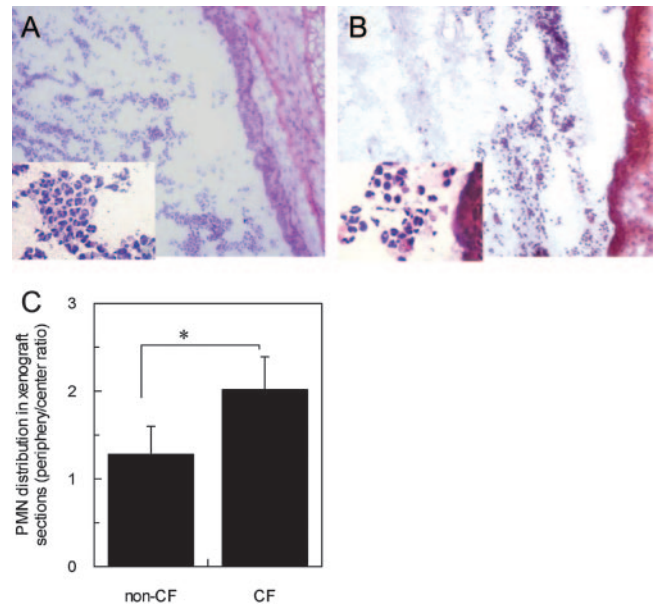


FIGURE 4. In vivo neutrophil migration into mucus plugs of non-CF and CF xenograft models. Neutrophil migration was examined with non-CF (2.5% mucus on normal airway epithelia) and CF (8% mucus on CF epithelia) airway models. *A*, Homogeneous neutrophil distribution throughout a mucus plug in a non-CF xenograft, $\times 10$ objective (*inset*, $\times 40$ objective). *B*, Periphery-dominant distribution of neutrophils in a CF xenograft, $\times 10$ objective (*inset*, $\times 40$ objective). *C*, Neutrophil distribution in normal and CF xenografts. Ratio of neutrophil numbers (periphery:center) was expressed for non-CF and CF xenograft models (mean \pm SE, $n = 6$ for non-CF and $n = 8$ for CF; *, $p < 0.05$).

Comparison of biochemical properties of CF vs normal mucus

Biochemical properties of CF and normal mucus were measured to determine whether there were any differences that might affect neutrophil migration. CF and non-CF mucins were compared for the quantity and characteristics of surface charge. Neither ion-exchange chromatography (Fig. 7A) nor agarose gel electrophoresis (Fig. 7B) detected differences in charge density or mass in CF vs normal mucins that might affect neutrophil migration.

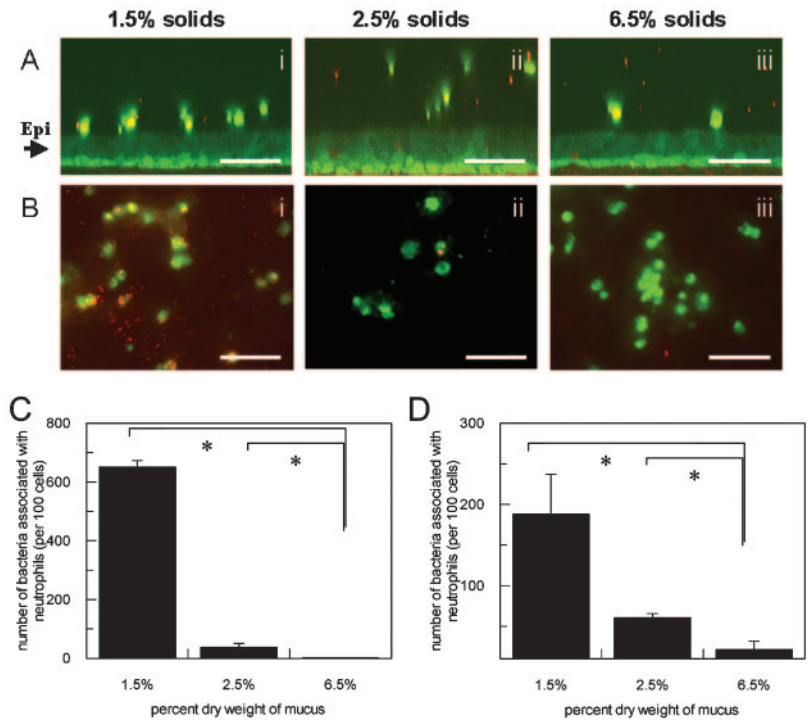
Neutrophil functions in CF-derived mucus of varying concentrations

Mucus from CF airway epithelial cultures was collected and concentrated to test whether there were any CF mucus-specific effects on neutrophil functions. Neutrophil chemotaxis in the gravitational and antigravitational directions was similar in CF to non-CF mucus (Fig. 7, C and D) (downward, ANOVA, $p = 0.00019$ and Tukey test, $p < 0.01$ for both 1.5 vs 2.5 and 1.5 vs 6.5%; upward, ANOVA, $p = 0.0045$ and Tukey test, $p < 0.05$ for 1.5 vs 2.5% and $p < 0.01$ for 2.5 vs 6.5%). Neutrophils migrated best in the gravitational direction in 1.5% solids mucus and not at all in 6.5% solids mucus, whereas mucus of 2.5% solids, but not 6.5% solids, supported neutrophil migration in the antigravitational direction. Bacterial killing by neutrophils was also repeated with CF mucus of varying concentrations on ciliated airway surfaces. Similar to the result with non-CF mucus, the number of bacteria was reduced by neutrophils over 2 h in 1.5 and 2.5% mucus but not in 6.5% mucus (Fig. 7E).

Discussion

Pathologic studies of lung disease in CF infants suggest that the formation of mucus plaques and plugs obstruct airways before the

FIGURE 5. Neutrophil capture of heat-killed bacteria in mucus on ciliated and nonciliated epithelial cultures. *A* and *B*, Distribution of neutrophils and bacteria after overnight incubation on nonciliated cultured epithelia. Neutrophils were stained with calcein (green), bacteria with tetramethylrhodamine (red), and epithelium (Epi) counterstained with calcein (green). *A*, x-z scanning images obtained by confocal microscopy ($\times 40$ oil immersion objective, N.A. = 1.3). Epi denoted by arrow. *i*, 1.5% mucus; *ii*, 2.5% mucus; and *iii*, 6.5% mucus. *B*, Images obtained by epifluorescence microscopy ($\times 40$ objective, N.A. = 0.6). *i*, 1.5% mucus; *ii*, 2.5% mucus; and *iii*, 6.5% mucus. All scale bars = 50 μm . *C* and *D*, Quantitation of neutrophil capture of heat-killed bacteria in mucus on ciliated and nonciliated epithelia. Neutrophils were incubated with fluorescently labeled, opsonized bacteria in mucus on nonciliated or ciliated airway epithelial cultures. The number of bacteria associated with neutrophils was counted on nonciliated (*C*) and ciliated (*D*) cultures (mean \pm SE; *, $p < 0.05$).



onset of bacterial infection (3). Recent studies have demonstrated that these mucus plaques and plugs are formed due to excessive ASL volume absorption, which depletes the lubricant periciliary layer and allows the concentrated mucus layer to adhere to airway surfaces (2, 4, 25). The mechanisms by which concentrated CF mucus produces infection are complex but include at least two

components: 1) failure to clear bacteria via the primary airways defense system, i.e., mucus clearance; and 2) reduction in secondary defense functions. Secondary airways defense mechanisms are multiple and interactive, but the goal of this study was to isolate and study the possible effects of mucus concentration on neutrophil defense mechanisms. Thus, we asked how efficiently neutrophils

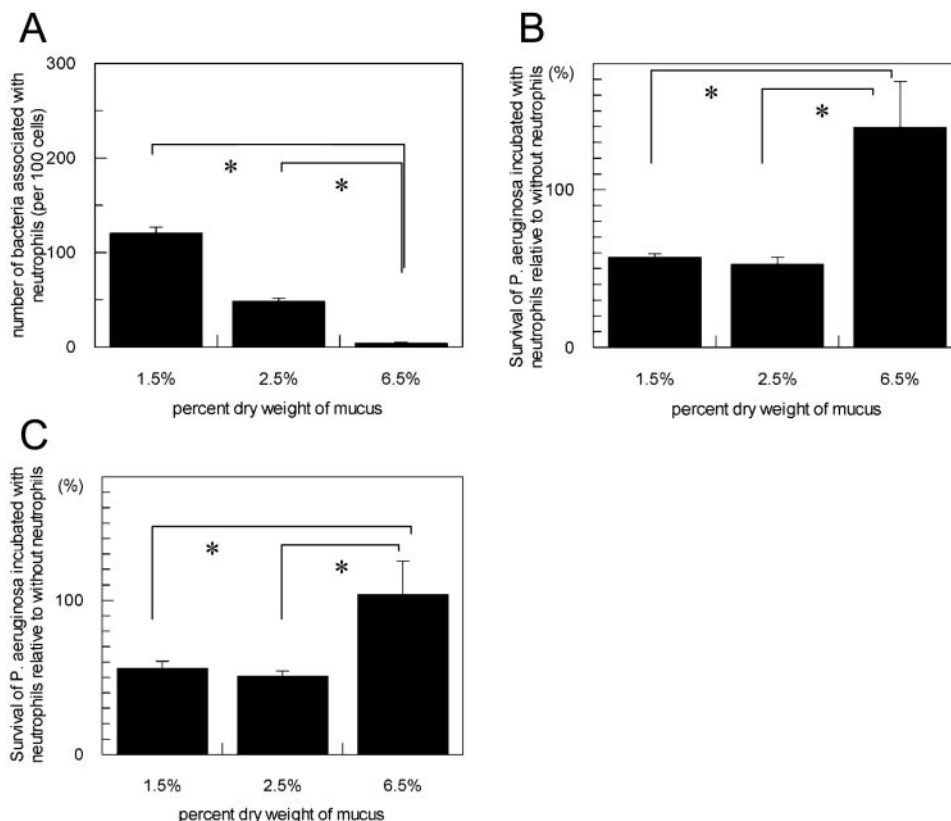
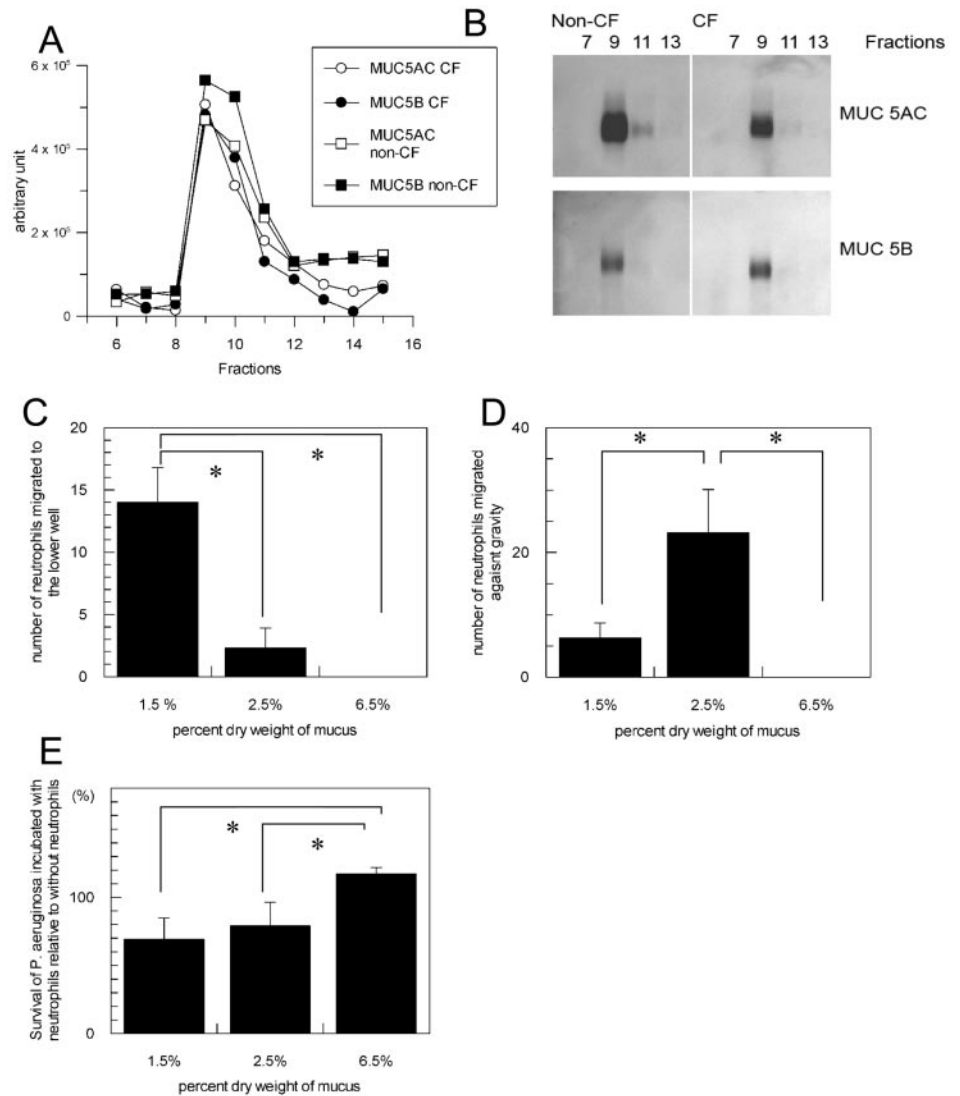


FIGURE 6. Neutrophil capture and killing of *P. aeruginosa* in mucus. Neutrophils were incubated with GFP-producing *P. aeruginosa* (PAO1 strain) in mucus of varying concentrations. The number of bacteria captured or survived was counted after 2-h incubation. *A*, The number of bacteria captured by neutrophils in mucus of varying concentrations (mean \pm SE; *, $p < 0.05$). *B*, Survival of bacteria on nonciliated epithelial culture surfaces with neutrophils ($\times 100$) in mucus of varying concentrations (mean \pm SE, $n = 4$; *, $p < 0.05$). *C*, Survival of bacteria on ciliated epithelial culture surfaces with neutrophils ($\times 100$) in mucus of varying concentrations (mean \pm SE, $n = 4$; *, $p < 0.05$).

FIGURE 7. Neutrophil functions in CF mucus of varying concentrations. Mucus of varying concentrations, derived from CF airway cultures, was prepared and compared with non-CF-derived mucus in terms of biochemical characteristics (A and B) and neutrophil functions (C–E). A, The charge density of the mucins present in the CF and non-CF secretions was compared by ion-exchange chromatography as described in *Materials and Methods*. Fractions were slot blotted and probed with mucin antisera to MUC5AC and MUC5B. B, The molecular dispersity of the mucins was compared by Western blotting after agarose gel electrophoresis (see *Materials and Methods* for details) performed on selected fractions 7, 9, 11, and 13 from the ion exchange. The direction of migration is as indicated. The same antisera were applied. C, Neutrophil migration in the direction of gravity (mean \pm SE, $n = 6$; *, $p < 0.05$; see Fig. 2A for comparison). D, Neutrophil migration against gravity (mean \pm SE, $n = 6$; *, $p < 0.05$; see Fig. 2B for comparison). E, Bacterial killing by neutrophils in varying concentration of CF mucus (mean \pm SE, $n = 4$; *, $p < 0.05$; see Fig. 6C for comparison).



move in normal mucus to capture/kill small numbers of either immotile or motile bacteria, as might be deposited in aerosol droplets onto airway mucus in vivo, and whether these capabilities are degraded in CF-like concentrated mucus.

The mucus layer covering airway surfaces is a non-Newtonian fluid with complex viscoelastic and structural properties. The structure and biophysical properties of mucus are imparted by the mucin macromolecules that normally comprise ~0.2–2% of solids content of this layer (Fig. 1A) and are organized as meshes with definable porosities (Fig. 1B). Mucus viscoelastic properties and the mesh organization appear well adapted under normal circumstances to trap and remove inhaled particles and bacteria from the lung. In CF, the abnormally concentrated mucus is predicted to have complex effects on viscoelastic properties and reduce the equivalent mesh size.

The selection of “normal” mucus, as indexed by concentration (percentage of solids) and CF-like concentrated mucus, is not straightforward because the shallow depth of ASL (<50 μm) makes sampling difficult. However, we estimated the percent dry weight (percentage of solids) of normal mucus at ~1.5–2.5% and of thick CF-like mucus at ~6–15%, based on data reported from cell culture (26), a mouse model (4), and human studies (27–33). Mucus of 1.5% solids may be less than the normal optimal concentration because the network of mucin fibers and transport were

heterogeneous (Fig. 1, B and C). However, this concentration could mimic the situation in small airways where there are few mucin-producing cells (34, 35) and little influx of mucus from more distal airways. The 2.5% mucus, which exhibited normal transport (Fig. 1C), may reflect the concentration of mucins that occurs in larger airways due to increased mucin secretion and ASL volume absorption (36). The higher concentration of mucus we operationally chose to mimic CF, 6.5%, likely represents the minimum concentration found in CF airways before infection (29, 30, 32, 33). Mucus transport assays demonstrated that 6.5% mucus was too thick to be transported by cilia, mimicking a key feature of CF mucus (2).

We estimated the size distribution of the mesh pores from the images acquired with confocal microscopy (Fig. 1B). There are limitations of this method. For example, the images obtained were thin (~0.5- μm depth) sections of a three-dimensional lattice. Therefore, all mucin fibers defining pore openings might not appear in a single image, which would overestimate pore size. In contrast, fibers seemingly defining pores may not actually interact with one another because of the poor z-axis resolution with confocal microscopy, which would underestimate pore size. However, the pore size measurements of mucus of different concentrations are consistent with our functional data. Most pore diameters of 1.5% mucus (>10 μm) and some of 2.5% mucus (1.6–14.1 μm)

were large enough for neutrophil (10–15 μm in diameter) penetration, whereas pores of 6.5% mucus were much smaller ($<0.2 \mu\text{m}$), i.e., smaller than a neutrophil or a bacterium.

Neutrophil motility in the presence of a chemoattractant varied dramatically as a function of mucus concentration and mesh pore size *in vitro* and *in vivo*. In *in vivo* xenograft models of mucus plugs, neutrophil migration was inhibited by thickened airway mucus, forcing neutrophils to stay near epithelial surfaces (Fig. 4). This result is compatible with the data from three-dimensional *in vitro* motility analysis.

In the modified chemotaxis chambers (Fig. 2), 1.5% mucus proved to be the optimal concentration for neutrophil migration in the direction of gravity, perhaps reflecting a minimum inhibition of neutrophil motility due to the large mesh pores relative to the size of neutrophils (Fig. 1B) and/or low mucus viscoelasticity. Interestingly, only a small number of neutrophils migrated with gravity through 2.5% mucus as compared with 1.5% mucus. We speculate that as the concentration of mucus increased from 1.5 to 2.5%, the density of mucin strands increased sufficiently to produce a population of pores sufficiently small to inhibit neutrophil entry into Transwell membrane pores. The absence of detectable motility in 6.5% mucus was likely caused by a mucus mesh pore size too small to allow neutrophil penetration.

In contrast, neutrophil migration in the antigravity direction was markedly faster in 2.5% mucus than in 1.5 or 6.5% mucus (Fig. 2B). We speculate that 1.5% mucus did not support migration because the mucus was too dispersed to provide anchoring supports (pore size $> 10 \mu\text{m}$) for upward motility, as reported for studies of three-dimensional neutrophil motility in collagen gels (10, 11). Mucus of 2.5% solids was filled with pores that were in the range of porous membranes of conventional chemotactic chambers (1–8 μm in diameter). We speculate that this pore size was sufficiently large to allow neutrophil penetration but small enough to prevent neutrophil slippage induced by gravity. We did not identify the mode of interaction of neutrophils with 2.5% mucus in this study, i.e., whether neutrophils dragged the mucin network, widened the pores of the network, or digested the mucin protein, as in collagen gels (37), or simply changed their shape to penetrate the mesh pores. The absence of neutrophil motility in the antigravity direction in 6.5% mucus, again, likely reflected an environment with a mesh pore size too small for neutrophils to enter (Fig. 1B).

Neutrophil chemokinesis on epithelial cell surfaces likely plays a significant role in capturing bacteria that escape the mucus blanket and sediment onto airway epithelial surfaces, especially in deciliated or goblet cell-metaplastic areas less well defended by mucus clearance. Neutrophils moved efficiently in search for bacteria on airway epithelia covered by mucus of 1.5% solids (Fig. 3B), reflecting little inhibition of lateral motility of neutrophils due to large mesh pores (Fig. 1Bi). Thick mucus (6.5% solids), with no detectable pores (Fig. 1Biii), inhibited lateral movement of neutrophils (Fig. 3B), preventing them from capturing bacteria sedimented or attached to airway epithelial surfaces. The relevance of this observation to *in vivo* situations was borne out in the xenograft experiments that showed concentration of neutrophils at the epithelial surfaces, which reflected inhibition of vertical movement of neutrophils, in 6.5 vs 2.5% mucus (Fig. 4).

In normal lung defense, inhaled bacteria are deposited and trapped in mucus. In diseases of failure to clear mucus, the chronic airways infection is localized within the mucus layer (5, 38). Despite this pathogenesis in the lung and similar infection of mucus by *H. pylori* in the stomach (39–41), there are a few studies of the role of neutrophil capture of immotile bacteria in a mucus environment. Therefore, we developed protocols to reproduce the *in vivo* environment in which neutrophil motility in three dimensions

within mucus was required to capture heat-killed bacteria. In 2.5% mucus, neutrophils moved both upward and laterally to capture bacteria (Fig. 5Ai). In contrast, in 1.5% mucus, neutrophils moved primarily laterally, requiring bacteria to sediment onto the epithelial surface for capture (Fig. 5Ai). Despite the restriction to vertical movement (Fig. 2Bii), neutrophils captured more bacteria in 1.5 than 2.5% mucus (Fig. 5C), reflecting the combined effects of higher lateral motility rates (Fig. 3B) and greater bacterial sedimentation that concentrated bacteria and neutrophils in the same plane (Fig. 5, Ai and Bi). In 6.5% mucus, the limited neutrophil motility in any direction and the absence of sedimentation of bacteria accounted for the virtual absence of bacterial capture. Similar relationships in neutrophil capture of immotile (heat-killed) bacteria were observed in the mucus of different concentrations on ciliated surfaces (Fig. 5D).

Finally, we tested whether neutrophil efficacy of capturing and killing motile bacteria in mucus was degraded in the mucus of abnormal concentration. In all three experimental approaches, 6.5% (concentrated) mucus inhibited neutrophil-mediated bacterial killing (Fig. 6). These data, plus those on bacterial capture (Fig. 5), led us to speculate that CF-like thickened mucus promotes the early acquisition of bacteria. These findings do not predict an early selection for a single type of bacteria, and indeed, CF infants are first infected by multiple different organisms, including both immotile (*Staphylococcus*) and motile (*H. influenza*, *Pseudomonas*) organisms. Only later, in the complex milieu of the CF chronically infected airway, will other factors, including mucus concentration and O_2 tension (5), allow *P. aeruginosa* to predominate (see below).

Our experiments performed in concentrated normal mucus were based on the hypothesis that concentration is the critical variable characterizing CF mucus (2, 4, 25). However, it is possible that other CF mucus-specific factors inhibited neutrophil functions as well. Differences between CF and non-CF mucus have been reported, including that CF mucins is more sulfated than non-CF mucins (42), which could affect fMLP diffusion and/or neutrophil migration. However, it is not clear whether these differences are derived from CF transmembrane conductance regulator-specific genetic defects or from chronic infection and inflammation (43).

Therefore, we compared mucin gene products and their charge secreted from normal and CF cultures. Ion-exchange chromatography and agarose electrophoresis revealed that no differences were detected in the negative charge of either mucin species (MUC5AC, MUC5B) secreted by CF and normal airway epithelia (Fig. 7, A and B). Thus, we could find no biochemical evidence that normal and CF mucins differed in the absence of infection.

To directly test neutrophil functions in CF-derived mucus, we next repeated three experiments using CF-derived mucus concentrated to 1.5, 2.5, and 6.5% solids (Fig. 7, C–E). Neutrophil chemotaxis and bacterial killing capacity in concentrated (6.5% solids) mucus and in 1.5 and 2.5% mucus from CF cultures mirrored precisely the functions of neutrophils in mucus obtained from normal cultures over the same range of mucus concentrations. Although we could not repeat all the experiments with CF mucus due to limited supply of CF bronchial cells, these results strongly indicate that mucus concentration, rather than source, inhibits neutrophil functions in airways.

The failure of neutrophils to kill bacteria in 6.5% mucus (normal or CF) may have implications not only for the early bacterial acquisition aspect of CF pathogenesis but also the persistent, excessive inflammatory response that characterizes later stages of the pathogenesis of CF airways disease. *P. aeruginosa* that proliferates in mucus causes inflammation by release of its own products and stimulation of host cellular cytokine production (44, 45). Components

of each system recruit neutrophils from the vascular circulation to airway lumens. If the mucus is too thick for neutrophil penetration to kill bacteria and resolve infection, activated neutrophils trapped at the edges of thickened mucus masses could cause adverse effects. For example, neutrophil elastase, released during “frustrated phagocytosis,” causes epithelial damage, Ig degradation, and mucus hypersecretion, all components of the uncontrolled inflammation that eventually leads to airway wall destruction (33, 46).

In conclusion, neutrophils migrate through normal respiratory mucus to search for and capture bacteria that have deposited in the mucus layer or have sedimented through this layer onto the airway epithelial surfaces. In contrast, the thick (concentrated) mucus in CF limits neutrophil motility and prevents bacterial capture and killing, likely contributing to both the early (acquisition) and chronic phases of airway bacterial infection. Thus, these studies strongly suggest that a key contribution of low ASL volume to the pathogenesis of CF airways disease is via the abnormally concentrated “thick” mucus layer characteristic of this disease that allows bacteria to evade the neutrophil component of lung defense.

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Disclosures

The authors have no financial conflict of interest.

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