

# Cystic Fibrosis Airway Epithelia Fail to Kill Bacteria Because of Abnormal Airway Surface Fluid

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## Summary

Despite an increased understanding of the cellular and molecular biology of the CFTR Cl<sup>-</sup> channel, it is not known how defective Cl<sup>-</sup> transport across airway epithelia causes chronic bacterial infections in cystic fibrosis (CF) airways. Here, we show that common CF pathogens were killed when added to the apical surface of normal airway epithelia. In contrast, these bacteria multiplied on CF epithelia. We found that bactericidal activity was present in airway surface fluid of both normal and CF epithelia. However, because bacterial killing required a low NaCl concentration and because CF surface fluid has a high NaCl concentration, CF epithelia failed to kill bacteria. This defect was corrected by reducing the NaCl concentration on CF epithelia. These data explain how the loss of CFTR Cl<sup>-</sup> channels may lead to lung disease and suggest new approaches to therapy.

## Introduction

The past few years have brought dramatic advances in our knowledge of the molecular and cellular basis of CF (for reviews see Collins, 1992; Riordan, 1993; Welsh et al., 1995). We now know that the disease is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation-regulated Cl<sup>-</sup> channel located in the apical membrane of involved epithelia. As well, much has been discovered about how CF-associated mutations disrupt protein function, thereby disrupting Cl<sup>-</sup> transport across CF epithelia.

Despite these advances, we do not understand the pathogenesis of CF lung disease, the major cause of morbidity and mortality. Lung disease is characterized by bacterial colonization and chronic airway infection. Many organisms can be involved, but *Pseudomonas aeruginosa* and *Staphylococcus aureus* are particularly prominent (Konstan and Berger, 1993). Chronic bacterial infections progressively destroy the lung and may ultimately lead to respiratory failure. Several hypotheses have been proposed to explain the pathogenesis of CF lung disease (Davis, 1993; Wine, 1995; Pilewski and Frizzell, 1995; Welsh et al., 1995). However, it has been difficult to relate the characteristic disease abnormality, bacterial colonization and infection of airways, to the

characteristic physiologic abnormality, defective trans-epithelial Cl<sup>-</sup> transport.

In other organs affected by CF, disease pathogenesis does not involve bacterial infections. For the sweat gland, pancreas, intestine, and male genital tract, plausible explanations of pathogenesis are based on defective transepithelial Cl<sup>-</sup> transport (Quinton, 1990; Welsh et al., 1995). Likewise, as suggested by Quinton (1984) over a decade ago, defective transepithelial electrolyte transport might somehow be responsible for the pathogenesis of airway infections. In airway epithelia, the loss of CFTR Cl<sup>-</sup> channel function, perhaps combined with a secondary defect in Na<sup>+</sup> transport, leads to abnormal transepithelial salt and fluid transport (Boucher et al., 1983; Jiang et al., 1993; Smith and Welsh, 1993; Smith et al., 1994). As a result, the composition of airway surface fluid is abnormal. Joris et al. (1993) and Gilljam et al. (1989) have shown that airway surface fluid from patients with CF has increased concentrations of Cl<sup>-</sup> and Na<sup>+</sup> when compared with that of normal subjects. But how do these abnormalities relate to airway infections?

## Results

### Normal but Not CF Epithelia Kill Bacteria Applied to the Apical Surface

Human airways are continually exposed to bacteria in ambient air (10<sup>3</sup>/m<sup>3</sup>) and to aspirated bacteria (DeKoster and Thorne, 1995; Huxley et al., 1978). Despite this exposure, the intrapulmonary airways remain sterile in healthy individuals. To begin our study of airway defenses, we asked what happens when bacteria are placed on normal airway epithelia. We used primary cultures of human airway epithelial cells as the model for these experiments. This model eliminates the antibacterial contribution of immune and inflammatory cells. We cultured the cells on permeable filter supports with air on the apical surface. Under these conditions, the cells form a continuous, polarized sheet that develops a transepithelial electrical resistance and that actively transports Na<sup>+</sup> and Cl<sup>-</sup> across the epithelium (Yamaya et al., 1992). Moreover, primary cultures of CF epithelia grown in this way manifest the CF defect in Cl<sup>-</sup> transport. Figure 1 shows that, under these conditions, epithelia develop a ciliated apical surface resembling that observed *in vivo* (Breeze and Wheeldon, 1977).

To mimic further the situation *in vivo*, we inoculated bacteria directly onto the air-covered apical surface of normal airway epithelia, using a small volume (20 nl). We added 30–300 colony-forming units (cfu) of *P. aeruginosa* to the surface and then placed the epithelia in a humidified cell culture incubator at 37°C. Within 24 hr after the bacteria were added to the apical surface, we recovered either no *P. aeruginosa* or fewer than we had added (Figure 2A). Moreover, the epithelia remained viable and uninfected for as long as they were maintained afterward (up to 3 weeks). We obtained strikingly different results with CF airway epithelia (Figure 2A); 24 hr

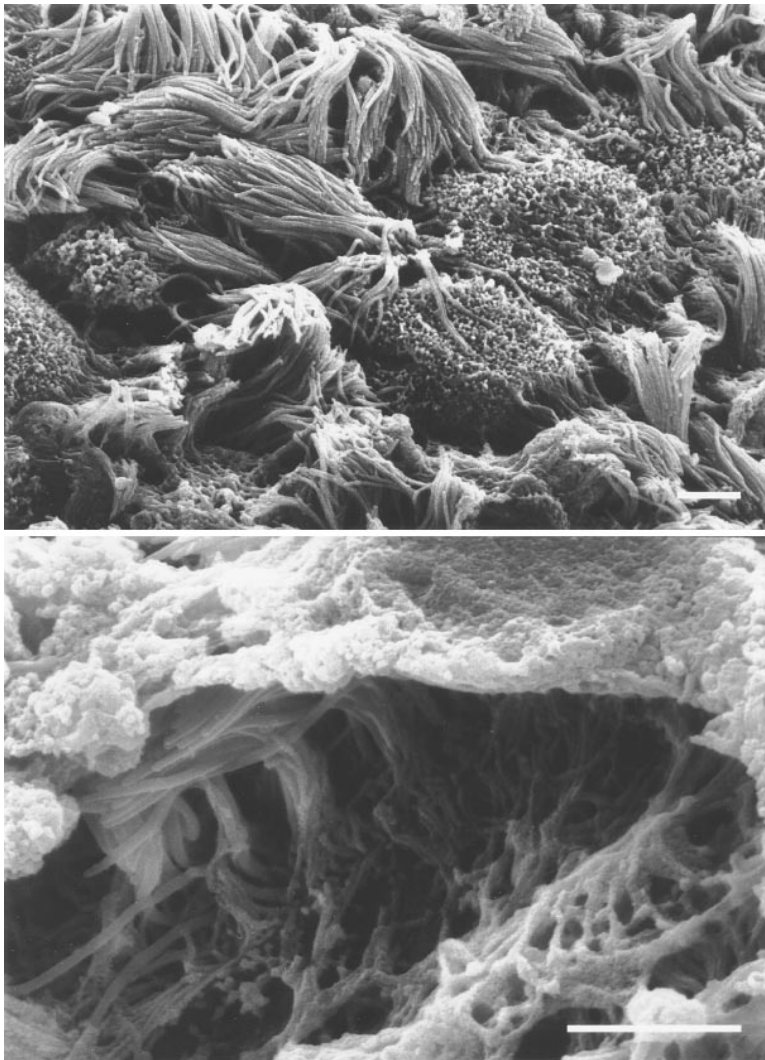


Figure 1. Scanning Electron Photomicrographs of the Apical Surface of Cultured Normal Airway Epithelium Studied 41 Days After Seeding

Top panel is low magnification. Bottom panel shows an area of epithelium with a thin layer of material covering the apical surface. The bar indicates 3  $\mu\text{m}$ .

after adding *P. aeruginosa* to the apical surface, we recovered more bacteria than had been added. When we expressed CFTR in CF epithelia using a recombinant adenovirus, the defect in killing *P. aeruginosa* was corrected (Figure 2A). Treatment with a related adenovirus vector expressing  $\beta$ -galactosidase had no effect. These data indicate that airway epithelia possess an anti-*Pseudomonas* activity that is dependent on CFTR.

Figure 2B shows that when we added up to  $10^3$  *P. aeruginosa* to normal epithelia, there were always fewer bacteria present 24 hr later. However, when we added more than  $10^3$  *P. aeruginosa*, a greater number of bacteria were recovered 24 hr later, suggesting that the antibacterial system was overwhelmed. Yet when even a small number of bacteria were added to the basolateral solution, there was always profuse growth (Figure 2B), suggesting that the antibacterial activity was localized to the apical surface of the epithelia. The rate of killing is shown in Figure 2C; 3 hr after addition of *P. aeruginosa* to the apical surface, the number of viable bacteria had decreased by about 50%. In contrast to airway epithelia, when we added less than 100 cfu of *P. aeruginosa* to the apical surface of Fisher rat thyroid epithelia, there was abundant growth ( $n = 15$ ; data not shown).

The airways of CF patients are colonized by many different bacteria; *S. aureus* is often one of the first organisms detected (Ramsey et al., 1991; Konstan and Berger, 1993). Figure 2D shows that normal epithelia also killed a methicillin-resistant clinical isolate of *S. aureus* added to the apical surface. Again, in contrast with normal epithelia, *S. aureus* multiplied on the surface of CF epithelia.

#### Normal and CF Airway Surface Fluids Contain Bactericidal Activity

Because bacteria were killed after addition to the small amount of fluid covering the apical surface of normal epithelia, we asked whether there was bactericidal activity in this airway surface fluid. We collected airway surface fluid by washing the apical surface with water and then added *P. aeruginosa* to the recovered fluid. Fluid recovered from normal airway surfaces killed *P. aeruginosa* (Figure 3A). Conversely, immediately after washing the apical surface, epithelia lost the ability to kill *P. aeruginosa* (Figure 3B). Airway surface fluid also killed *Escherichia coli* and clinical isolates of *P. aeruginosa* and methicillin-resistant *S. aureus* (Figure 3A). These

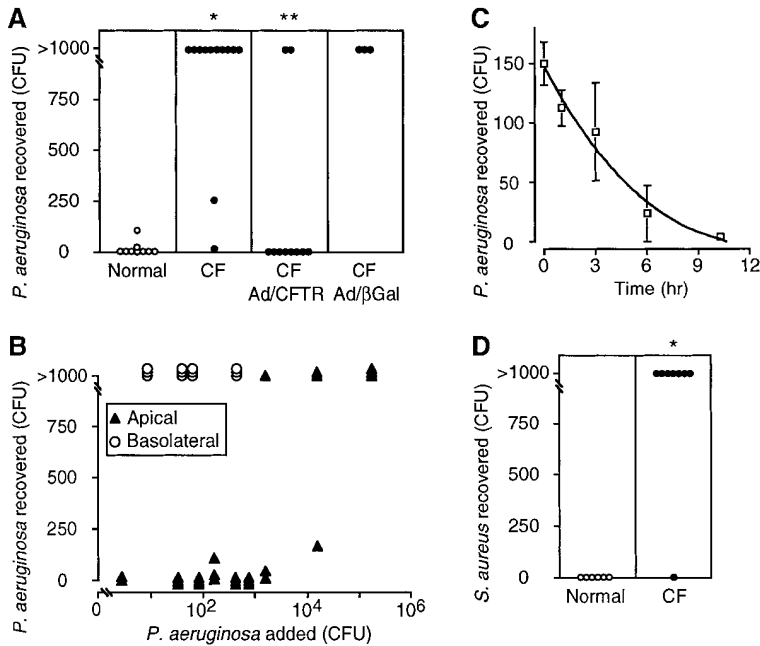


Figure 2. Killing of Bacteria by Airway Epithelia

(A) *P. aeruginosa* recovered from normal and CF epithelia 24 hr after addition of 30–300 cfu. CF epithelia were treated with Ad2-CFTR-8 or Ad2- $\beta$ -gal-2, as indicated, 3–4 days before addition of *P. aeruginosa*. Each data point is from an individual epithelium. Asterisk indicates  $p < 0.0001$  compared with normal epithelia; double asterisk indicates  $p < 0.003$  compared with CF epithelia without Ad2-CFTR-8.  
 (B) *P. aeruginosa* recovered after addition of indicated number of bacteria to the apical surface (closed triangles) or basolateral solution (open circles). Data points indicate the total cfu recovered from each epithelium.  
 (C) Effect of duration of incubation on *P. aeruginosa* recovered after addition of  $137 \pm 30$  cfu to the apical surface of normal airway epithelia. Data are mean  $\pm$  standard error of the mean;  $n = 3$  epithelia at each timepoint.  
 (D) *S. aureus* recovered 24 hr after addition of  $70 \pm 8$  cfu to the apical surface of normal or CF epithelia. Asterisk indicates  $p < 0.02$ .

results suggest that airway surface fluid contains broad-spectrum bactericidal activity.

The broad spectrum of bactericidal activity suggested that surface fluid might contain a defensin-like factor (Lehrer et al., 1993; Martin et al., 1995). Microfiltration experiments indicated that the bactericidal factor appeared to be smaller than 10 kDa; airway surface fluid that had passed through a Microcon-10 filter (Amicon) had anti-*Pseudomonas* activity equal to that of unfiltered airway surface fluid ( $n = 12$ ). In addition, boiling the fluid for 10 min did not abolish its activity as compared with unboiled fluid ( $n = 12$ ). A low molecular mass

and heat stability are characteristic of defensins (Lehrer et al., 1993; Martin et al., 1995).

Because bacteria multiplied on the surface of CF epithelia, we asked whether the bactericidal factor was missing in CF. Interestingly, airway surface fluid collected in water from either normal or CF epithelia killed *P. aeruginosa* (Figure 3C). This finding indicates that CF epithelia do not lack a bactericidal factor. Therefore, we hypothesized that CF epithelia fail to kill *P. aeruginosa* (see Figure 2A) because the composition of airway surface fluid is altered so as to inhibit the activity of a bactericidal factor.

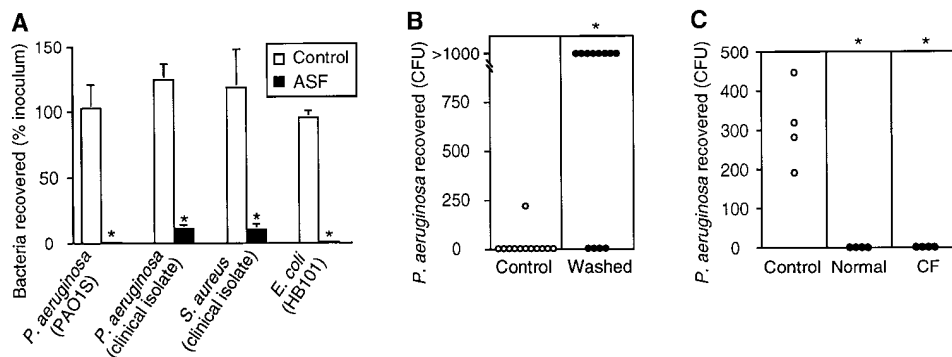


Figure 3. Killing of Bacteria by Airway Surface Fluid

(A) Bacteria recovered after incubation in airway surface fluid (ASF) collected in water from normal epithelia. *P. aeruginosa* (PAO1S) ( $49 \pm 9$  cfu), a clinical isolate of *P. aeruginosa* ( $69 \pm 19$  cfu), a clinical isolate of *S. aureus* ( $58 \pm 9$  cfu), or *E. coli* HB101 ( $351 \pm 115$  cfu) were incubated in water (control) or airway surface fluid in water (ASF) at  $37^\circ\text{C}$  for 3 hr. Data are mean  $\pm$  standard error of the mean;  $n = 3$  for each point. Asterisk indicates  $p < 0.006$  compared with control.

(B) *P. aeruginosa* recovered 24 hr after addition to normal epithelia, as described in legend to Figure 2, or to epithelia in which the apical surface had been washed before addition of bacteria. Asterisk indicates  $p < 0.01$ .

(C) *P. aeruginosa* ( $665 \pm 75$  cfu) was added to water alone (control) or to airway surface fluid collected in water from CF or normal airway epithelia and incubated at  $37^\circ\text{C}$  for 3 hr. Similar results were obtained in three other sets of experiments in which 25–70 cfu were added. Asterisk indicates  $p < 0.001$  compared with control.

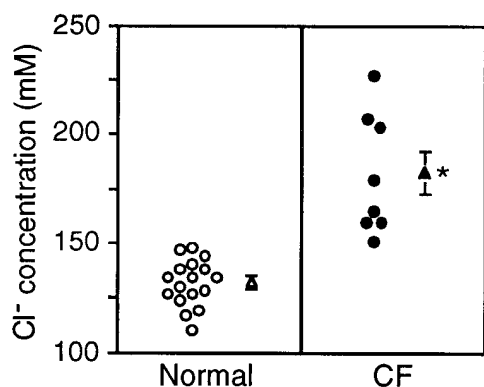


Figure 4. Concentration of  $\text{Cl}^-$  in Airway Surface Fluid Collected from Nasal Epithelium of CF and Normal Subjects

CF,  $n = 8$ ; normal,  $n = 17$ . Each point is the value from an individual subject; mean  $\pm$  standard error of the mean are indicated. Asterisk indicates  $p < 0.001$  compared with normal.

#### Airway Surface Fluid from CF Epithelia Has an Abnormally Increased $\text{Cl}^-$ Concentration

Joris et al. (1993) and Gilljam et al. (1989) used bronchoscopy to obtain airway surface fluid from the trachea and main stem bronchi of normal and CF subjects. They found that normal fluid had  $\text{Cl}^-$  concentrations of  $84 \pm 9$  mM and  $85 \pm 54$  mM, respectively, whereas CF fluid had higher  $\text{Cl}^-$  concentrations of  $129 \pm 5$  mM and  $170 \pm 79$  mM, respectively. To confirm this difference, we measured the  $\text{Cl}^-$  concentration in airway surface fluid obtained from the nasal mucosa; we used nasal mucosa because the function and histology of the epithelium is similar to that of intrapulmonary airways, and it is easily accessible. Like the earlier reports, our data showed that the  $\text{Cl}^-$  concentration in CF fluid ( $182 \pm 10$  mM) was higher than in normal fluid ( $132 \pm 3$  mM) (Figure 4). Evaporation or methodological differences or both may account for the fact that we found higher  $\text{Cl}^-$  concentrations in nasal fluid than were reported in tracheal fluid (Joris et al., 1993; Gilljam et al., 1989). However, the important point is that  $\text{Cl}^-$  concentrations were significantly higher in CF. This may occur because the loss of CFTR  $\text{Cl}^-$  channels prevents  $\text{Cl}^-$  from accompanying  $\text{Na}^+$  absorption, much as occurs in CF sweat ducts (Quinton, 1990, 1994).

#### An Increased NaCl Concentration Inhibits Bactericidal Activity in Airway Surface Fluid

To learn whether the electrolyte concentration could affect bactericidal activity, we removed airway surface fluid with solutions containing different concentrations of NaCl and tested the ability of the fluid to kill bacteria in vitro. As the concentration of NaCl increased, anti-*Pseudomonas* activity decreased (Figure 5A). Similar results were obtained with clinical isolates of *P. aeruginosa* and *S. aureus* and with *E. coli* (Figures 5A and 5B). These data indicate that increased electrolyte concentrations reduce bactericidal activity.

Bactericidal activity was also present when we collected airway surface fluid with 140 mM NaCl (instead of with water) and then diluted the fluid to a NaCl concentration of 47 mM before adding *P. aeruginosa* (Figure

5C). These data indicate that although the electrolyte composition affects bactericidal activity, it does not affect the ability to recover the bactericidal factor from the airway surface.

#### Reduction of the NaCl Concentration Allows CF Epithelia to Kill *P. aeruginosa*

These results suggest that CF epithelia produce a bactericidal factor that fails to kill bacteria applied to their apical surface because the surface fluid has an abnormally high salt concentration. Thus, we reasoned that if we altered the electrolyte concentration on the airway surface, we would alter bactericidal activity. To test this hypothesis, we applied a small amount of solution (60  $\mu\text{l}$ ) with a known salt concentration to the apical surface of normal and CF airway epithelia and then added *P. aeruginosa*. When *P. aeruginosa* was added to normal epithelia covered with a solution containing a low  $\text{Cl}^-$  concentration, the bacteria were killed (Figure 6A). This result is the same with direct addition of *P. aeruginosa* to the airway surface (see Figure 2A). However, with a high salt concentration on the apical surface of normal epithelia, the bacteria multiplied. Thus, increasing the salt concentration caused normal epithelia to behave like CF epithelia. Most importantly, when the salt concentration was reduced, bacteria placed on CF epithelia were killed (Figure 6B). Thus, a low salt concentration allowed CF epithelia to kill *P. aeruginosa*.

Scanning electron photomicrographs confirmed these results. We added *P. aeruginosa* to CF epithelia covered with a thin layer of solution containing either 182 mM  $\text{Cl}^-$  or 92 mM  $\text{Cl}^-$ . Within 48 hr after their addition to epithelia with a high salt concentration, we observed *P. aeruginosa* on the apical surface (Figure 7A). In contrast, we could not find bacteria on CF epithelia when the salt concentration was low (Figure 7B).

#### Discussion

These data provide a link between the physiologic hallmark of CF, defective transepithelial  $\text{Cl}^-$  transport, and the clinical hallmark of CF, airway infections with CF pathogens such as *P. aeruginosa* and *S. aureus*. The results suggest that airway epithelia secrete a bactericidal substance into the thin layer of fluid covering the apical surface, where its activity depends on a low salt concentration. In CF epithelia, loss of CFTR  $\text{Cl}^-$  channels produces an abnormally high salt concentration in the airway surface fluid, which reduces bactericidal activity. When the salt concentration is lowered, CF epithelia can kill *P. aeruginosa*.

Bactericidal activity in the airway surface fluid may be the first line of defense that protects the lung from bacteria and helps maintain a sterile intrapulmonary environment. Loss of this activity could explain lung disease in patients with CF as follows. Bacteria deposit on the airway surface after inhalation and aspiration. Normally, they would be killed by bactericidal activity in airway surface fluid. However, our data suggest that this system is impaired in CF. As a result, a second line of defense, neutrophils and macrophages, may kill the bacteria and release cytokines that recruit additional



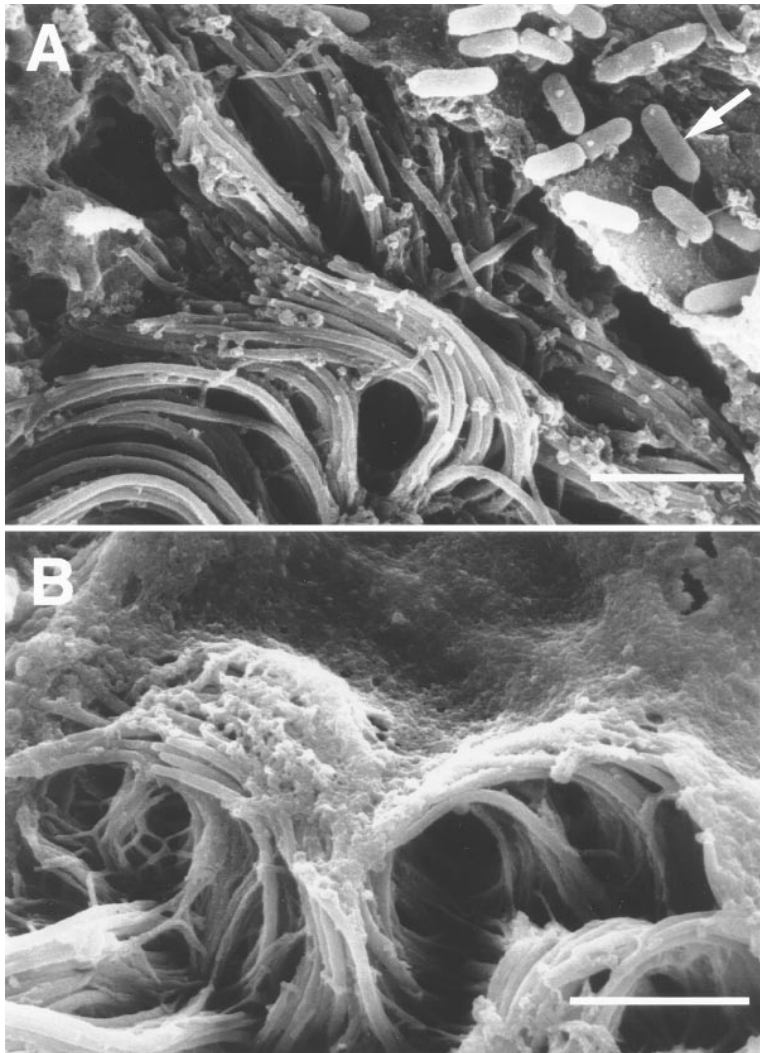


Figure 7. Scanning Electron Photomicrographs of the Surface of CF Epithelia

*P. aeruginosa* (PAO1S  $459 \pm 47$  cfu) was added 48 hr before processing. Epithelia were covered with  $60 \mu\text{l}$  of a solution containing either  $182 \text{ mM Cl}^-$  (A) or  $92 \text{ mM Cl}^-$  (B), as described in legend to Figure 6. Bacteria were usually found on a thin film of material with a mucus-like appearance. Cilia are visible beneath the thin film of material. Arrow indicates *P. aeruginosa*. Bar indicates  $3 \mu\text{m}$ .

destruction, or both. Yet, without invoking other proposals, our data showing that electrolyte composition affects bacterial survival provide an explanation for why CF airways are not maintained as a sterile environment.

We speculate that the bactericidal factor produced by airway epithelia may be a defensin-like molecule, because it has several properties characteristic of such factors (for reviews see Lehrer et al., 1993; Martin et al., 1995): it is a low molecular mass, heat-stable substance that has broad-spectrum bactericidal activity, and killing is dependent on salt concentration. In addition, expression of a defensin has been detected in bovine airway epithelial cells (Diamond et al., 1993). Identification of the bactericidal factor and elucidation of its mechanism of action will provide additional insights into local pulmonary defense mechanisms and could lead to the development of more effective bactericidal agents.

Our results link the molecular defect in CFTR  $\text{Cl}^-$  channels to the pathogenesis of CF lung disease. More importantly, the data suggest novel assays for evaluating potential treatments and new approaches to therapy. For example, measurements of salt concentration and bactericidal activity may be clinically relevant assays for

determining the effectiveness of potential therapeutic interventions. It will be interesting to learn how pharmaceuticals designed to alter electrolyte transport, such as amiloride and UTP (Knowles et al., 1990, 1991), affect electrolyte composition. In the case of gene therapy, knowledge of the relationship among the percentage of cells that express CFTR, the amount of CFTR expression per cell, and correction of the abnormal composition of airway surface fluid may help guide therapeutic trials. The data also raise the intriguing possibility that new interventions designed to correct the abnormally high salt concentration in CF fluid could be of benefit in treating or preventing airway infections in people with CF.

#### Experimental Procedures

##### Culture of Epithelia

Airway epithelial cells were isolated from nasal, tracheal, and bronchial epithelia from 5 CF and 14 normal (non-CF) people. Cells were seeded on collagen-coated, semipermeable membranes ( $0.6 \text{ cm}^2$ ) and grown at the air-liquid interface as previously described (Yamaya et al., 1992). Because we were establishing primary cultures, for 2–4 days after seeding, the culture medium contained either 100 mU/ml penicillin plus 100  $\mu\text{g/ml}$  streptomycin or the combination

of penicillin, streptomycin, 50 µg/ml gentamicin, 40 µg/ml tobramycin, 15 µg/ml colymycin, 125 µg/ml ceftazidime, and 2 µg/ml flucanazole. Similar results were obtained with epithelia initially treated with either antibiotic mixture. After antibiotic treatment, the basolateral solution was replaced five times over 48 hr with antibiotic-free medium, and the apical surface was washed three times over 48 hr with antibiotic-free phosphate-buffered Ringer's solution. Thereafter, the antibiotic-free medium was changed at 2–3 day intervals. Epithelia were studied 8–45 days after seeding and showed bactericidal activity even after many changes of the basolateral solution and many washes over time of the apical solution. All epithelia used showed no leakage of fluid from the basolateral to the apical surface, and paired monolayers had transepithelial resistances of  $>500 \Omega \times \text{cm}^2$ . Epithelia were prepared for scanning electron microscopy using standard techniques.

In some studies, epithelia were treated with recombinant adenovirus to express CFTR. Previous studies have shown that Ad2-CFTR-8 (moi = 50) used under these conditions corrects the fluid and electrolyte transport defects in CF epithelia (Zabner et al., 1994; J. Zabner and M. J. W., unpublished data). As a control, some epithelia were exposed to Ad2-β-gal-2, which expresses β-galactosidase. As an additional control, one epithelium in each experiment was used to study the electrical properties to determine that cAMP-stimulated Cl<sup>-</sup> transport was corrected by treatment with Ad2-CFTR-8. Fisher rat thyroid epithelia were cultured as described (Sheppard et al., 1994).

#### Bacterial Stains and Culture Conditions

We used several bacteria, as follows: a laboratory strain of *P. aeruginosa* (PAO1S), a spontaneously occurring streptomycin-resistant mutant of the naturally ampicillin-resistant strain PAO1 (isolated by Dr. C. Cox); *E. coli* HB101 (a streptomycin-resistant strain); a clinical isolate of *P. aeruginosa* (a mucoid strain from a CF patient); and a methicillin-resistant clinical isolate of *S. aureus*. Bacteria were grown overnight in Luria broth. They were then washed three times in water, centrifuged at  $4000 \times g$  for 15 min, and diluted in water to an appropriate density. The number of cfu were also measured directly in triplicate by plating 20 nl on Luria agar and counting colonies.

#### Application of Bacteria to Epithelia

A 20 nl drop of the bacterial suspension was applied to the apical or basolateral surface, and epithelia were then incubated at 37°C in a humidified cell culture incubator. After 24 hr, bacteria were recovered from epithelia by washing the apical surface with 60 µl of water (1–3 washes). Values greater than 1000 cfu were not determined, and data are reported as  $>1000$  cfu. However, in some cases in which absolute counts were made,  $>10^6$  cfu were present when we report  $>1000$  cfu.

The 20 nl drop of bacteria was placed on the center of the epithelial monolayer and probably did not spread over the entire epithelium; therefore, the relationship among the number of bacteria added, the area exposed, and the amount of antibacterial activity could not be determined.

#### In Vitro Studies of Bactericidal Activity

Airway surface fluid was collected by washing the apical surface of epithelia with 60 µl of water or a NaCl solution, as indicated. After pooling the recovered fluid, bacteria (in 20 nl of water) were added to 30 µl of the fluid and incubated at 37°C for 3 hr. Under these in vitro conditions with airway surface fluid in water, *P. aeruginosa* was killed rapidly, with a 50% decrease in viability in approximately 30 min (data not shown). The volume of airway surface fluid collected from the epithelia could not be determined but is very small, probably less than 1 µl. Bactericidal activity could not be attributed to the cell culture medium, because bacteria multiplied when added to the basolateral medium (Figure 2B) or when added to a small amount of culture medium diluted in water (data not shown). As described above, bactericidal activity could be removed from the epithelia by washing the surface. However, 24–48 hr after the apical surface was washed, epithelia recovered the ability to kill bacteria, and we could once again collect bactericidal activity. In fact, we

could collect bactericidal activity on several occasions over many days from a single epithelium.

Evidence that epithelia were not damaged by collection of surface fluid includes the following: after washing, epithelia retained their transepithelial resistance and electrolyte transport properties; there was no leakage of fluid from the basolateral to the apical surface; and 24 hr after washing, we could again recover bactericidal activity. Moreover, as described above and in Figure 5C, bactericidal activity could be recovered either with water or with a NaCl solution.

#### Collection and Analysis of Airway Surface Fluid from Normal and CF Subjects

Airway surface fluid was collected from 8 CF and 17 normal subjects. Before collection, subjects wore a nose clip for 5 min to prevent breathing through the nose. Immediately after removal of the nose clip, a 0.32 cm<sup>2</sup> filter (66213, Gelman Sciences, Ann Arbor, MI) was gently applied to the inferior surface of the inferior turbinate. After 5 s, the filter was withdrawn and immediately immersed in mineral oil to prevent evaporation. The mineral oil was not water saturated. Fluid was extracted from the filter as follows. The bottom of the microcentrifuge tube holding the filter in mineral oil was perforated, and the tube was placed into a larger tube containing mineral oil. The tandem tubes were centrifuged at  $174,000 \times g$  for 15 min to transfer the fluid into the larger tube without exposing it to air; the filter remained in the smaller microcentrifuge tube. Fluid samples collected from both nostrils of a subject were pooled, and Cl<sup>-</sup> concentrations were measured by chloridometry (Labconco Corp., Kansas City, MO). Control samples containing known Cl<sup>-</sup> concentrations were separated, stored, and analyzed in an identical manner; in the control samples, Cl<sup>-</sup> concentrations were always within 5 mM of the original concentration.

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