Efficient killing of inhaled bacteria in ΔF508 mice: role of airway surface liquid composition

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McCray, Paul B., J R., Joseph Zabner, Hong Peng Jia, Michael J. Welsh, and Peter S. Thorne. Efficient killing of inhaled bacteria in ΔF508 mice: role of airway surface liquid composition. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L183–L190, 1999.—Cystic fibrosis mice have been generated by gene targeting but show little lung disease without repeated exposure to bacteria. We asked if murine mucosal defenses and airway surface liquid (ASL) Cl− were altered by the ΔF508 cystic fibrosis transmembrane conductance regulator mutation. Naive ΔF508 −/− and +/+ mice showed no pulmonary inflammation and after inhaled Pseudomonas aeruginosa had similar inflammatory responses and bacterial clearance rates. We therefore investigated components of the innate immune system. Bronchoalveolar lavage fluid from mice killed Escherichia coli, and the microbial activity was inhibited by NaCl. Because β-defensins are salt-sensitive epithelial products, we looked for pulmonary β-defensin expression. A mouse homolog of human β-defensin-1 (termed “MBD-1”) was identified; the mRNA was expressed in the lung. Using a radiotracer technique, ASL volume and Cl− concentration ([Cl−]) were measured in cultured tracheal epithelia from normal and ΔF508 −/− mice. The estimated ASL volume was similar for both groups. There were no differences in ASL [Cl−] in ΔF508 −/− and normal mice (13.8 ± 2.6 vs. 17.8 ± 5.6 meq/l). Because ASL [Cl−] is low in normal and mutant mice, salt-sensitive antimicrobial factors, including MBD-1, may be normally active.

cystic fibrosis transmembrane conductance regulator; Pseudomonas aeruginosa; defensin; cystic fibrosis

ONE APPROACH TO understanding the pathogenesis and pathophysiology of cystic fibrosis (CF) is to develop animal models that reflect the disease. Toward that end, CF mouse models have been generated using gene targeting to disrupt the murine cystic fibrosis transmembrane conductance regulator (CFTR) locus by homologous recombination (7, 15, 40, 43) and by introducing specific human mutations into the equivalent mouse loci, including ΔF508 (9, 59) and G551D (13). However, although intestinal disease manifestations are prominent in these animals, there is remarkably little evidence of lung disease in “CF mice” maintained under normal housing conditions. Explanations put forward to account for the low incidence of lung infection in CF mice include the presence of alternative Cl− transport pathways (8, 25) and the presence of modifier genes (45).

Several layers of defenses in the normal lung help prevent infection from inhaled or aspirated microorganisms. These include the mechanical filtering of particulates that occurs in the nasal airway, the trapping of particulates in mucus, and mucociliary clearance. Respiratory epithelia also secrete a number of protein and peptide products that are important in the innate mucosal immunity (17, 19, 44, 50). In addition, macrophages and neutrophils may participate in the clearance of microorganisms from the lung, usually at the cost of some degree of inflammation (44). In humans with CF, the early onset inflammation characterized by neutrophilia and proinflammatory cytokines in bronchoalveolar lavage (BAL) precedes chronic infection (1, 2, 34). In contrast to the characteristic lung disease of humans with CF, mouse models have had more variable pulmonary manifestations. Initial reports of CFTR null mice showed little evidence of lung disease (9, 40, 59). A later report by Davidson and colleagues (12) demonstrated that, with repeated bacterial challenges, it is possible to find evidence of decreased clearance of inhaled bacteria and persistent inflammatory disease. Van Heeckeren et al. (55) found that CFTR null mice showed more inflammation and morbidity when challenged with agar beads coated with Pseudomonas aeruginosa. Kent and colleagues (32) found that an inbred congenic strain of CFTR null mice spontaneously developed lung disease; however, unlike CF in humans, the disease was primarily alveolar. Thus with exposures to large bacterial loads or alteration of the genetic background, CF mouse models may develop lung disease.

The goal of these studies was to learn if the lungs of mice homozygous for the ΔF508 mutation show any differences in baseline markers of pulmonary inflammation or develop lung disease when challenged with aerosolized bacteria. We approached these issues by performing BAL studies on naive mice before and after exposure to aerosolized P. aeruginosa. We found no significant differences between homozygous ΔF508 mice and their heterozygous littermates.

When we found little difference between the groups, we undertook further studies to investigate components of the innate immune system in mice. Recent studies indicate that human airway surface liquid (ASL) contains salt-sensitive antimicrobial factors that may be important in lung defenses (23, 48). These factors are secreted products of epithelia, exhibit broad spectrum activity, and may be inactive in CF ASL due to its elevated salt concentration (22, 23, 31, 48, 57). Thus altered electrolyte transport in CF epithelia may increase the salt concentration in ASL and impair...
the activity of innate mucosal antimicrobial factors. Therefore, we evaluated some of these aspects in the ΔF508 mouse model, focusing on the effects of inhaled bacteria on lung inflammation, the antimicrobial properties of mouse ASL, the expression of β-defensins in the lung, and the electrolyte composition of ASL.

MATERIALS AND METHODS

Animals. ΔF508 mice were generated by gene targeting in ES cells as previously reported (59). The genetic background of the mice is a cross of the C57Bl/6 and 129 strains. The animals were housed in normal conditions (not pathogen free) before study and were genotyped using tail DNA PCR as previously reported (59). To ensure that mice were correctly genotyped during the inhalation challenge and postinhalation procedures, an identifying microchip was implanted under the skin of each animal, and animals were regenotyped at the end of study (Mini Tracker; Avid, Norco, CA). For the inhalation challenge, two groups of animals were studied, ΔF508 homozygotes (−/−) and ΔF508 heterozygotes (+/−). The rationale for the use of ΔF508 heterozygotes as controls in the studies was as follows. There is no evidence that heterozygous CFTR null mice or ΔF508 mice have a pathological or physiological phenotype. Likewise, with the exception of abnormalities of stimulated sweat secretion, humans heterozygous for CFTR mutations do not have a disease phenotype.

Inhalation exposure system. Mice were exposed by inhalation to aerosolized P. aeruginosa in a 40-liter glass whole body exposure chamber for a period of 4 h with a modification of previously described methods (37, 51). For these studies, the P. aeruginosa strain ATCC no. 10145 was used (American Type Culture Collection, Manassas, VA). The bacterial solution for generating the aerosol was obtained by placing one cryovial of laboratory-cultured and lyophilized P. aeruginosa in 50 ml of sterile pyrogen-free saline in a 37°C shaker bath. A nebulizer was supplied with temperature-controlled, filtered air and was operated at 101.5 kPa gauge pressure. The exposure chamber exhaust rate was set at 20.0 l/min and was metered with a rotometer calibrated against a primary reaction chamber exhaust rate. Bacteria were grown at 30°C in Luria-Bertani medium, centrifuged, and resuspended at a concentration of 10⁷ cells/ml in 10 mM potassium phosphate, pH 7.2, with 1% Luria-Bertani medium. Bacteria (10⁷) were then incubated with mouse BAL fluid in 96-well plates (Optiplate; Packard Instruments) for 4 h, and luminescence was measured with a microtiter dish luminometer (Anthus). To assess the salt sensitivity of the BAL fluid activity, the assays were performed in the presence of 0 or 150 mM added NaCl.

Cloning the murine β-defensin-1 cDNA. The human β-defensin-1 (HBD-1) mature peptide sequence (DHYNCVSSG-GQCLYSACPIFTKIQGTCYRGKAKCCKS) was used to screen the GenBank database for expressed sequence tags (ESTs) using the NCBI BLAST program. The search identified six identical murine ESTs with homology to HBD-1. Four clones contained the entire 210-bp open reading frame for the putative murine β-defensin-1 (MBD-1). Primer sequences were designed to the putative MBD-1 sequence and used to clone the cDNA from mouse kidney using RT-PCR. Mouse kidney RNA was treated with DNase I (RNasefree; Promega, Madison, WI) for 1 h to remove genomic DNA before the RT reaction, phenol-chloroform extracted, and precipitated. One microgram of total RNA was reverse transcribed using the GeneAmp PCR reagents (Perkin-Elmer, Norwalk, CT). Moloney murine leukemia virus RT, and a poly(A) reverse primer. The resultant cDNA was used as a template in the PCR. Primer sequences were as follows: forward primer sequence, CACATCCTCTCTGCACTCTGGACCC; reverse primer, CCATCGCTGCTTTATGCTTACCC. Each reaction contained 0.15 µM primers, 2 mM Mg²⁺, and the 20-µl RT reaction product in a total volume of 100 µl. After an initial denaturation step (95°C for 3 min), 25 cycles of annealing (60°C for 30 s) and extending (72°C for 30 s) were performed. The predicted 287-bp MBD-1 PCR product (bp 39 to 248 of the cDNA) was isolated and cloned into the PCR Script vector (Stratagene, La Jolla, CA), and the identity was confirmed by DNA sequencing.

RT-PCR of MBD-1. Total RNA was isolated using the single-step RNAzol method (5). One microgram of total RNA was reverse transcribed by random hexamer primers using the SuperScript transcription system (GIBCO BRL) according to the manufacturer’s instructions. First-strand MBD-1 cDNA was amplified by PCR as described above. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in the same reaction using the following primers: GAPDH forward primer, GTCACTGGT-GACCTGAACCT; GAPDH reverse primer, AGGCTGCTACAT- GCAACTG. A 25-µl aliquot of the PCR product was electrophoresed on a 2% agarose gel and visualized with ethidium bromide.

Cell culture. Mouse tracheal epithelial cells were cultured at the air-liquid interface using a modification of previously described methods (56, 58). Tracheal epithelial cells were plated on collagen-coated 0.6-cm² Millicell HA filter inserts at a density of 5 × 10⁴ cells/cm². The nutrient medium consisted
of DMEM-F-12 with 2% Ultroser G (48). Viability of the cultures was confirmed visually by verifying that the epithelia maintained a dry apical surface in culture and by documenting the bioelectric properties of the cells. Under these conditions, the epithelia differentiate and develop a ciliated apical surface (56, 58). All cultures used in measurements of ASL fluid and electrolyte composition had a transepithelial resistance (Rt) >500 Ω·cm² (measured by EVOM; World Precision Instruments). The mean Rt for ΔF508 −/− cultures was 664.67 ± 102.5 Ω·cm² (n = 12) and for normal epithelia was 613.33 ± 57.83 Ω·cm² (n = 12). The differences were not statistically significant (P = 0.4). The amiloride-sensitive short-circuit current for ΔF508 −/− and normal cells was not significantly different and ranged between 5 and 10 µA/cm² (n = 12 for ΔF508 −/− and normal epithelia). The tracheae from 10 mice were combined to make one epithelial cell preparation.

Measurement of ASL volume and Cl− concentration by radiotracer technique. Because knowledge of the salt concentration at the air interface is critical to understanding CF airway disease, we developed a new radiotracer method to measure ASL Cl− concentration (Cl−); see Ref. 57). To measure [Cl−] in ASL, we added 36Cl− to the basolateral medium together with 3H2O. After the tracer content of ASL reached equilibrium (48 h), we removed ASL by rinsing the apical surface with 100 µl of medium. The basolateral medium was also sampled, and its [Cl−] was measured.

RESULTS

Naive animals have normal BAL profiles. In infants and children with CF, it appears that a defect in innate immunity contributes to the chronic airway inflammation and infection that is a clinical hallmark of the disease. Asymptomatic infants with CF may have elevations in BAL neutrophils and proinflammatory cytokines that precede the onset of symptoms (2, 34). We tested the hypothesis that ΔF508 −/− mice housed under normal conditions from birth develop pulmonary inflammation by measuring BAL cell counts and cytokine levels. Under such housing conditions, animals will be chronically exposed to low levels of inhaled bacteria. Naive ΔF508 −/− animals were compared with littermates heterozygous for the ΔF508 mutation. As shown in Table 1, there were no significant differences in the percentage of neutrophils or the proinflammatory cytokines IL-1, TNF-α, or IL-6 between the BAL fluids of ΔF508 −/− or +/- animals. This result suggests that there are no differences between animal groups in pulmonary bacterial clearance mechanisms, which include both filtering mechanisms and innate immunity.

Normal and ΔF508 mice clear inhaled bacteria at similar rates. The chronic inflammation in infants and children with CF is characteristically neutrophilic. Because we detected no differences between ΔF508 −/− and +/- animals with low-level exposure to inhaled bacteria, we tested the hypothesis that the ability to clear an inhaled bacterial challenge is impaired in ΔF508 −/− mice compared with heterozygote littermates. For these experiments, mice were exposed to aerosolized P. aeruginosa in a whole body exposure chamber for 4 h. In this exposure procedure, the airborne concentration of P. aeruginosa was 3.3 × 10⁸ CFU/m³ for an estimated lung deposition of 1 × 10⁶ CFU/animal (see MATERIALS AND METHODS). At this dose of inhaled bacteria, we assume the mechanism of bacterial clearance included both innate and cellular responses. After the exposure, groups of animals were killed at 5, 24, and 72 h, and BAL studies were performed. As shown in Fig. 1A, both ΔF508 −/− and +/- mice developed marked BAL neutrophilia after bacterial inhalation. This was greatest at 5 h after onset of P. aeruginosa inhalation and gradually declined over 72 h. Quantitative BAL bacterial colony counts, shown in Fig. 1B, demonstrated that both groups had similar amounts of P. aeruginosa recovered at 5 h, and the bacteria were rapidly cleared by 24 h after exposure in both groups. The BAL neutrophilia was accompanied by a rise in IL-6 and TNF-α at 5 h, although there were no significant differences between the groups (data not shown). From these studies, we conclude that the pulmonary inflammatory responses and bacterial clearance after inhalation challenge with P. aeruginosa are equivalent for ΔF508 −/− and +/- mice.

Murine BAL fluid exhibits salt-sensitive antimicrobial activity. The observation that ΔF508 −/− and +/- mice cleared bacteria equally efficiently and had no demonstrable elevations in proinflammatory cytokines or neutrophils in BAL under normal housing conditions suggests two possible explanations. First, murine pulmonary antimicrobial factors are salt insensitive, such as the protegrins from pig neutrophils (60). Second, murine ASL might maintain a low NaCl concentration even in the face of CFTR mutations. To address the first possibility, we isolated BAL fluid from normal mice and tested its ability to kill bacteria in the presence or absence of added NaCl. As shown in Fig. 2, murine BAL fluid killed E. coli in a concentration-dependent manner. When the BAL samples were tested in the presence of 150 mM NaCl, the antimicrobial activity was lost. Thus murine BAL fluid, like human ASL (48), exhibited antimicrobial activity that was inhibited by salt.

Table 1. BAL profiles of naive ΔF508 −/− and +/- mice housed under normal conditions

<table>
<thead>
<tr>
<th>n</th>
<th>Cells</th>
<th>PMN, %</th>
<th>IL-1, pg/ml</th>
<th>TNF-α, pg/ml</th>
<th>IL-6, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508 −/+</td>
<td>4</td>
<td>17,000 ± 6,623</td>
<td>0.33 ± 0.33</td>
<td>7.5 ± 10.6</td>
<td>17.5 ± 10.6</td>
</tr>
<tr>
<td>ΔF508 −/+</td>
<td>5</td>
<td>31,000 ± 2,920</td>
<td>0.25 ± 0.25</td>
<td>7.5 ± 17.5</td>
<td>12.9 ± 10.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. PMN, polymorphonuclear neutrophils; IL, interleukin; TNF-α, tumor necrosis factor-α; BAL, bronchoalveolar lavage. There were no significant differences in baseline BAL cell numbers, %neutrophils, and levels of IL-1, IL-6, or TNF-α. All cytokines were below the limit of detection except for IL-6 in one ΔF508 −/− animal.
Murine lung expresses MBD-1. ASL contains many antimicrobial factors, including the proteins lysozyme (21) and lactoferrin and peptides such as the β-defensins (20). Recent evidence suggests that low-molecular-weight peptides such as the β-defensins may play an important role in pulmonary defenses in humans (23, 47, 48). We wondered whether epithelial β-defensins similar to those reported in bovine (14) and human (23, 26, 38) lungs were also expressed in the murine lung. Although mice are known to express a class of intestinal defensins termed cryptdins (30, 41), no MBD had been identified at the time of these studies. Using the HBD-1 sequence as a query (4), we searched the GenBank dbest data base of ESTs and found an MBD homolog. The MBD-1 cDNA clone consisted of a 210-bp open reading frame, predicted to encode a 69-amino acid protein (shown in Fig. 3A). The cDNA clone isolated from mouse kidney was identical to the MBD-1 cDNA reported by Huttner and colleagues (29), and the predicted mature peptide sequence was ~55% identical to HBD-1 (4, 38). To determine the tissue distribution of MBD-1 mRNA, we performed RT-PCR (Fig. 3B). MBD-1 mRNA was expressed most abundantly in kidney and testes, with transcripts also detected in lung, brain, bladder, and stomach.

ASL [Cl\textsuperscript{-}] in normal and ΔF508 / – mice is similar. In humans, one proposed effect of CFTR mutations on airway epithelia is an impaired ability to absorb NaCl (22, 23, 31, 48, 57). This leads to an elevation in ASL NaCl concentration and may inhibit the activity of salt-sensitive antimicrobial factors secreted by epithelia, including β-defensins. Measurements of the ASL composition in normal and CF subjects suggest that the NaCl concentration in CF ASL is elevated compared with that in normal mice (22, 23, 31, 57). We performed experiments to address this issue in mice. Using a radiotracer technique, we measured [Cl\textsuperscript{-}] in ASL of primary cultures of tracheal epithelia from ΔF508 / – mice and wild-type mice. We added radiolabeled Cl\textsuperscript{-} and H\textsubscript{2}O to the basal media of confluent cultures of tracheal epithelial cells. After 48 h, the labeled ions reached equilibrium between the mucosal and basal solutions, and measurements were made. As shown in

![Figure 1](http://ajplung.physiology.org/)  
**Fig. 1.** ΔF508 / – and + / – mice show similar responses to inhaled bacteria. Animals were exposed to aerosolized Pseudomonas aeruginosa for 4 h as described in MATERIALS AND METHODS and were killed at the indicated intervals postexposure, and bronchoalveolar lavage (BAL) was performed. A: BAL polymorphonuclear neutrophil (PMN) counts. B: BAL colony-forming units (CFU) for P. aeruginosa (CFU/ml). Results are means ± SE.

![Figure 2](http://ajplung.physiology.org/)  
**Fig. 2.** Antimicrobial activity of murine BAL fluid determined by Escherichia coli luminescence assay. Serial dilutions of BAL were added to the assay buffer as indicated on the x-axis. BAL from normal mice exhibited antimicrobial activity that was inhibited in the presence of 150 mM NaCl. Results are means ± SE for BAL analysis from 4 mice. RLU, relative light units.
RESISTANCE TO LUNG INFECTION IN ΔF508 MICE

DISCUSSION

This study shows that ΔF508 −/− and +/+ mice housed under normal conditions show no significant inflammation on BAL analysis, suggesting equivalent abilities to clear inhaled bacteria. Furthermore, when challenged with inhaled P. aeruginosa, both groups developed similar increases in BAL fluid neutrophilia and cytokines. These inflammatory responses were transient, and over 24–72 h, bacteria were cleared from the lungs of both groups with similar rapidity. Thus we were unable to demonstrate any differences between ΔF508 −/− and +/+ mice in their inflammatory responses and clearance of inhaled bacteria.

Several mouse models of CF have been generated, including CFTR null mice and mice with specific mutations, in the hope that these animals would prove to be useful for the study of CF lung disease (7, 9, 13, 15, 40, 43, 59). A striking feature of some models is the severity of the gastrointestinal disease and minimal evidence of lung disease (9, 40, 59). Kent et al. (33) reported the pathological findings on CFTR null mice surviving >100 days and found no pulmonary abnormalities. Snouwaert and co-workers (49) reported minor pulmonary abnormalities consisting of increases in goblet cells and mucus in cells of the upper airways of CFTR null mice surviving to adulthood. In one animal, marked goblet cell hyperplasia, mucus obstruction, and inflammatory cells were noted. No differences between CFTR null and normal mice were noted in bacterial clearance after repeated exposure to Staphylococcus aureus over a 1-mo period (49). In contrast, Davidson and colleagues (12) found that, on repeated exposure to S.
that, in non-CF epithelia, the ASL NaCl concentration was reduced compared with serum (10). We recently used a radiotracer method to measure ASL NaCl concentrations in mice (6, 7, 15, 40, 43) and in mice homozygous for the AF508 mutation (9). The epithelial sodium channel is also expressed in the lung and presumably accounts for the amiloride-sensitive Na+ conductance across murine airway epithelia (11, 28). However, CFTR-mediated Cl− transport may be less important in murine airway epithelia because there are alternative Cl− transport pathways available. Compared with human airways, some strains of mice show evidence of significant Ca2+–activated Cl− channel activity that might compensate for the absence of CFTR (6, 8, 24, 25). Indeed, Grubb and colleagues (24, 25) reported that respiratory epithelia of CFTR null mice exhibit increased Ca2+-activated Cl− channel activity compared with that in normal mice. Perhaps murine CFTR plays a minor role in regulating the composition of ASL because alternative Cl− channels provide a pathway for NaCl absorption and facilitate the maintenance of a low salt concentration. We speculate that one explanation for the lack of significant lung disease in AF508 mice, and perhaps in other CF mouse models, is that the low salt environment of murine ASL allows salt-sensitive components of their pulmonary mucosal defenses, including MBD-1, to function normally.

**NOTE ADDED IN PROOF**


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