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*J Immunol* 2000; 165:3941-3950; doi: 10.4049/jimmunol.165.7.3941

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Role of Cystic Fibrosis Transmembrane Conductance Regulator in Pulmonary Clearance of *Pseudomonas aeruginosa* In Vivo

Zissis C. Chroneos,*  Susan E. Wert,*  Jennifer L. Livingston,*  Daniel J. Hassett, † and Jeffrey A. Whitsett2*†

Cystic fibrosis (CF) is a fatal genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) that is commonly associated with chronic pulmonary infections with mucoid *Pseudomonas aeruginosa* (PA). To test the hypothesis that CFTR plays a direct role in PA adhesion and clearance, we have used mouse lines expressing varying levels of human (h) or mouse (m) CFTR. A subacute intratracheal dose of $3 \times 10^8$ bacteria was cleared with similar kinetics in control wild-type (WT) and transgenic mice overexpressing hCFTR in the lung from the surfactant protein C (SP-C) promoter (SP-C-hCFTR<sup>+/−</sup>). In a second series of experiments, the clearance of an acute intratracheal dose of $1.5 \times 10^7$ PA bacteria was also similar in WT, hemizygous SP-C-hCFTR<sup>+/−</sup>, and bitransgenic gut-corrected FABP-hCFTR<sup>+/−</sup>-mCFTR<sup>−/−</sup>, the latter lacking expression of mCFTR in the lung. However, a small but significant decrease in bacterial killing was observed in lungs of homozygous SP-C-hCFTR<sup>−/−</sup> mice. Lung pathology in both WT and SP-C-hCFTR<sup>+/−</sup> mice was marked by neutrophilic inflammation and bacterial invasion of perivascular and subepithelial compartments. Bacteria were associated primarily with leukocytes and were not associated with alveolar type II or bronchiolar epithelial cells, the cellular sites of SP-C-hCFTR<sup>+/−</sup> transgene expression. The results indicate that there is no direct correlation between levels of CFTR expression and bacterial clearance or association of bacteria with epithelial cells in vivo.

results are associated with the complex genetic background of the transgenic mice, dietary differences, and the potential role of alternative chloride channels in mice, which are not activated in humans (3). Thus it remains unclear whether bacterial clearance or inflammatory responses are directly related to the levels of CFTR in lung cells in vivo.

Clearance of PA in animal models is associated with a prompt inflammatory response characterized by neutrophilic influx and cytokine production. Neutrophils facilitate phagocytic clearance of bacteria, whereas cytokines modify the bactericidal activities of alveolar macrophages and neutrophils to maximize bacterial clearance and limit excessive tissue-damaging inflammation (15–18). However, CF patients fail to eradicate bacteria despite a high neutrophilic influx, and it is thought that the resultant inflammatory response is the major contributor to the progressive deterioration of lung function (1). Transgenic mice with a null mutation in the C5a receptor also fail to clear PA bacteria from the lungs despite a high influx of neutrophils, suggesting that abnormal functional activation of inflammatory cells may contribute to decreased bacterial clearance and excessive inflammatory responses (19). Recent studies implicate CFTR in the orchestration of a balanced inflammatory response of the epithelium. For example, CF epithelial cells have diminished production of TNF-α (20), IL-10 (21), RANTES (22), and glutathione (23), whereas IL-8 is expressed at high levels (24, 25), suggesting that CFTR influences an important epithelial-based component in the inflammatory response of the lung. Studies in resistant and susceptible mice indicate that early secretion of TNF is associated with a prompt neutrophilic influx and enhanced bacterial clearance (16, 26, 27). Effective bacterial uptake and killing by lung leukocytes during infections requires the presence of surfactant proteins A (SP-A) and D (SP-D) and complement components (28). The levels of SP-A and SP-D are diminished in CF (29). A recent study indicates an important role of mannose binding protein (MBP), a serum homolog of SP-A and SP-D, in the severity of CF disease. Patients expressing MBP alleles defective in opsonic clearance of bacteria are more severely affected than those with normal MBP alleles (30). Whether a similar allelic component in the outcome of CF exists for SP-A and SP-D is not yet known.

In addition to an apparent linkage between CFTR and heterogeneous components of humoral and cell-mediated immune mechanisms in the human lung, it is hypothesized that CFTR facilitates epithelial clearance of bacteria as an epithelial receptor for the core oligosaccharide component of PA and Salmonella typhi (31–35). This functional activity of CFTR is inferred from in vitro studies with epithelial cell lines expressing normal or mutant CFTR proteins and the ability of mAbs to amino acid sequence 103–117 of CFTR to block in vivo clearance of bacteria. However, other in vitro studies do not support a role of CFTR in the epithelial uptake of bacteria and indicate that epithelial invasion by PA requires access and attachment of bacteria to the basolateral epithelial surface, which may occur during epithelial repair and polarization (36–40). Attachment to epithelia may occur in part via the asialo-GM1 receptor that appears to be increased on epithelial cells as a result of CFTR dysfunction or epithelial cell repair (36, 40).

To address whether CFTR contributes to the clearance of bacteria from the lung we have used transgenic mice that express varying levels of mCFTR or hCFTR in respiratory epithelial cells, in which the hCFTR mRNA is expressed under the control of the human SP-C promoter (41). To correlate bacterial clearance with the level of CFTR expression we have also used the FABP-hCFTR+/−-mCFTR+/− transgenic mice that do not express the murine CFTR gene, lack CFTR mRNA in the lung, and do not suffer from nutritional effects of CF-related gastrointestinal dysfunction (8). In these studies, bacterial clearance from the lungs of transgenic mice was not directly related to the presence, absence, or increased expression of CFTR mRNA in vivo.

Materials and Methods

Animal husbandry

Generation of transgenic mice expressing the hCFTR protein in the lungs of FVB/N mice (SP-C-hCFTR+/−) was previously described (41). The J4 SP-C-hCFTR+/− mouse line expressing high levels of the hCFTR protein in distal airways in alveolar type II and bronchiolar epithelial cells was used in all experiments. To generate heterozygous (SP-C-hCFTR+/−) and littermate control (WT) mice, transgenic SP-C-hCFTR+/− mice were bred with WT FVB/N mice (Charles River Breeding Laboratories, Wilmington, MA). Bacterial clearance in commercially purchased FVB/N mice was similar to WT littermate control mice from our colony. Generation of gut-corrected bitransgenic null mutant mice (FABP-hCFTR+/−-mCFTR−/−) was described previously (8). These mice were obtained after mating FVB/N transgenic mice expressing the hCFTR gene in the gut under the influence of the rat FABP promoter with CFTR-null mutant mice (8, 42). The null mutant mice (mCFTR−/−) originally generated by Snouwaert et al. (42) were backcrossed into the FVB/N background for two generations before mating with FABP-hCFTR+/−-mCFTR−/− mice. The offspring from the bitransgenic mice with the appropriate transgenic phenotype (FABP-hCFTR+/−-mCFTR−/−) have been propagated continuously in our animal facility and were used in these studies. The FABP-hCFTR+/−, mCFTR−/− strain is predominantly FVB/N, with an additional mixed genetic component from C57BL/6, 129/SvEv, BALb/c, and DBA/2 strains (10). For the purpose of this study and because the study of Cressman et al. (10) demonstrated similar airway disease and bacterial clearance between the original mCFTR−/− and control mice in each different genetic background, we did not backcross the bitransgenic mouse further into the FVB/N background. Animals were housed and studied under Institutional Animal Care and Use Committee-approved protocols in the animal facility of the Children’s Hospital Research Foundation (Cincinnati, OH). Male and female mice of −8–10 wk old were studied. The body weights of WT and the various transgenic mice were similar. The mean weight of WT, SP-C-hCFTR+/−, and FABP-hCFTR+/−-mCFTR−/− mice was 26.5 ± 1.05, 26.04 ± 0.64, and 25.18 ± 0.56, respectively.

Preparation of bacteria

PA strain FRD1, a mucoid CF isolate (43) and strain PA01 (44), a nonmucoid, wound isolate were used in these studies. Bacterial stocks were maintained at −70°C in LB medium (Luria-Bertani broth, 10 g tryptone, 5 g yeast extract, 5 g NaCl/L) containing 20% glycerol. Single bacterial colonies on LB agar plates were used to inoculate 4 ml of LB and grown to stationary phase for 16–18 h with vigorous aeration at 37°C. Bacteria were clarified by centrifugation at 5500 × g for 15 min, washed, and suspended in HBSS (0.38 mM Na2HPO4, 0.44 mM KH2PO4, 0.4 mM MgSO4·7H2O, 0.49 mM MgCl2·7H2O, 0.126 mM CaCl2·2H2O, 5.34 mM KCl, 0.137 M NaCl, and 4.5 mM glucose). Bacterial titers were determined by enumeration of CFU on LB agar plates after serial dilution in PBS.

Bacterial clearance

After intratracheal inoculation of bacteria (28), lungs were harvested at 2 to 24 h postinfection, homogenized in 2 ml of Dulbecco’s PBS (8.098 mM Na2HPO4, 0.44 mM KH2PO4, 0.49 mM MgCl2·7H2O, 0.9 mM CaCl2·2H2O, 2.68 mM KCl, and 0.137 M NaCl), and the CFU was determined corrected for growth of serially diluted homogenates on LB agar plates. Inocula of 3 × 103 or 1.5 × 105 CFU were administered as indicated. The subacute inoculum of 3 × 106 CFU of the mucoid PA strain FRD1 was essentially cleared from the lungs of all mice by 24 h postinfection with less than 1000 CFU detected at 24 h. The acute inoculum of 1.5 × 107 CFU was the highest dose of the mucoid PA strain FRD1 that all mice survived in the first 24 h postinfection. In appropriate experiments bacteria were quantitated in lung lavage and postlavage tissue homogenates at indicated time points after infection for 24 h. Bronchoalveolar lavage (BAL) was accomplished after sequential instillation of PBS in 1-ml portions, collecting a total of 4 ml of fluid. Cytokine measurements were performed in BAL after a low spin centrifugation to remove alveolar cells. Less than 1% of measured cytokines were present in the cell pellet.

Lung pathology

Lungs were fixed by inflation using a tracheal cannula at 25 cm of pressure with 4% paraformaldehyde prepared in PBS. The lungs were removed and
were assembled in a standard 20°C aqueous solution. The percentage of neutrophils recruited to the lung was determined after cytospin centrifugation (Shandon, Pittsburgh, PA) of an alveolar cell pellet obtained from BAL as described above. Cells were stained with the Diff-Quick stain (Fisher, Pittsburgh, PA) to visualize and distinguish alveolar macrophages from neutrophils. Cell numbers were scored in five different microscopic fields and the percentage of neutrophils was determined from combined numbers obtained from different fields.

Expression of CFTR mRNA

The relative level of expression of the hCFTR transgene in SP-C-hCFTR H/H mice and the endogenous murine CFTR was determined by RT-PCR. All mRNA preparations were treated with DNaseI (Life Technologies, Rockville, MD) to remove genomic DNA contamination. Total lung mRNA from WT and SP-C-hCFTR H/H was isolated using a commercial kit (Qiagen, Chatsworth, CA). To synthesize first strand cDNA, 2 μg of mRNA was primed with 1.5 μg of oligo(dT) (Roche, Giuff-Oberfrick, Switzerland). Reverse transcription was carried out at 42°C for 1 h in the designated first strand reaction buffer supplemented with 10 mM DTT, 0.5 mM dNTP (Roche), 20 U of RNAsin (Promega, Madison, WI), and 100 U of Superscript II reverse transcriptase (Life Technologies) in 20 μl final reaction volume. The reaction was terminated at 95°C for 5 min and stored frozen at −20°C. The mCFTR and hCFTR cDNAs were amplified separately by PCR from 1 μl of RT reaction using Taq DNA polymerase (PE Applied Biosystems, Foster City, CA) The human primers were 5′-AGT GAGTATGCAGAAATAGTG-3′ and 5′-TCCACAGCTCCAAATTC CATGAGG-3′ that correspond to nucleotides 4055–4660 in the human CFTR (45). The mouse primers were 5′-AATGGAAAGATGAA GAAATATGG-3′ and 5′-TTCACACTCTCTAAGTCGACGC-3′ that correspond to nucleotides 4055–4930 of the mouse cDNA. PCR reactions were assembled in a standard 20 μl reaction volume containing 2 μl 10× Opti-prime buffer 12 (Stratagene, La Jolla, CA), 0.4 μM each primer, 0.25 mM dNTP, 0.25 μM of each primer, 4 μl of 5× PCR dye, and 0.5 U of AmpliTaq polymerase. The thermal cycler (PE Applied Biosystems) parameters were set at 94°C for 5 min, 1 cycle; 94°C for 30 s, 58°C for 1 min, 5 cycles; 72°C for 7 min, 1 cycle. To obtain an estimate of the number of each CFTR transcript, a standard curve was generated by PCR amplification of hCFTR or mCFTR using known amounts of control CFTR plasmids. For mCFTR plasmid pMC1 containing the entire hCFTR transcript and mCFTR plasmid p8AC containing exon 13 of mCFTR was used as standard (46). For hCFTR or mCFTR transcripts in the lungs of SP-C-hCFTR H/H mice was estimated separately from standard curves as described in Materials and Methods. Error bars are means ± SE with p = 0.008 for hCFTR vs mCFTR (n = 3).

Quantitation of cytokines

The levels of TNF-α and IL-1β were measured in lung homogenates using commercially available kits according to the manufacturer’s directions (R&D Systems, Minneapolis, MN).

Statistical analysis

Differences among treatment groups were assessed by ANOVA. Significance of the differences was accepted for p < 0.05.

Results

The goal of this study was to determine whether the levels of CFTR influenced bacterial clearance and inflammation in response to pulmonary PA infection in vivo. We used adult transgenic mice with increased lung-specific expression of the hCFTR protein in alveolar type II and bronchiolar epithelial cells (SP-C-hCFTR H/H or H/−) (41) and CFTR knockout mice in which hCFTR was selectively expressed in the gastrointestinal tract under the control of the rat FABP promoter (FABP-hCFTR H/−-mCFTR H/−) (8). The relative expression of hCFTR and mCFTR mRNAs in the lungs of SP-C-hCFTR H/− mice was assessed by RT-PCR. Expression of hCFTR was increased ~7.7-fold compared with mCFTR in SP-C-hCFTR H/− mice (Fig. 1). This finding is consistent with previous studies demonstrating a 10-fold higher expression of hCFTR mRNA than the endogenous mCFTR estimated from Northern blot analysis in SP-C-hCFTR H/− mice (41). The FABP-hCFTR H/−-mCFTR H/− mouse line does not express detectable hCFTR or mCFTR mRNA in the respiratory tract (8).

Intratracheal administration of PA

Control and transgenic mice were intratracheally inoculated with bacteria at acute or subacute levels of infection, and the number of bacteria and the inflammatory response of the lung were measured over a 24-h period. In initial experiments, we established the acute and subacute intratracheal infection dose using PA strain FRD1, a mucoid clinical isolate from a CF patient (43) in 8-wk-old WT mice. All mice survived the acute intratracheal dose of 1.5 × 107 CFU within the experimental period of 24 h, and all mice completely cleared the subacute infective dose of 3 × 106 CFU by 24 h. Most studies were performed using the mucoid FRD1 strain. In a more limited set of experiments, the clearance of the nonmucoid PA01 strain (44) was compared with the clearance of FRD1 to examine the effect of mucoidy in bacterial clearance and inflammation. Infection-induced cellular inflammation was evaluated in BAL and lung tissue sections, and the cytokines TNF-α and IL-1β, measured by ELISA, were used as the soluble inflammatory indicators.

Bacterial clearance in WT and transgenic mice

The clearance of a subacute (3 × 106 CFU) infection with FRD1 was assessed in heterozygous SP-C-hCFTR H/− mice and WT littermate control mice at 2, 3.5, 6, and 24 h postinfection (Fig. 2). Furthermore, the number of bacteria was determined in lung lavage and postlavage lung homogenates to assess whether the level of CFTR expression influenced the association of organisms with lung tissue. A similar rapid decline in bacterial numbers was observed in

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FIGURE 2. Kinetics of bacterial clearance after a subacute pulmonary infection with PA FRD1. Eight-week-old littermate WT (□) and SP-C-hCFTR+/+ (■) heterozygous transgenic mice were infected intratracheally with 3 × 10^6 CFU of PA FRD1. Bacterial numbers were determined in alveolar lavage and postlavage tissue homogenates at 2 (n = 5), 3.5 (n = 4), and 6 h (n = 5) postinfection as described in Materials and Methods. A, Sum of CFU obtained in lavage and postlavage tissue homogenate, representing an estimate of bacterial counts in whole lung. Less than 1000 total CFU was detected at 24 h. B, Total CFU present in postlavage tissue homogenate. C, Total CFU present in alveolar lavage. Error bars are means ± SE. All p values comparing statistical differences between WT and SP-C-hCFTR+/+ were >0.05.

both transgenic and littermate control mice between 2 and 6 h, and the infection was essentially cleared by 24 h, with <1000 CFU/lung being detected in lungs of either strain at that time (Fig. 2). Approximately 60% of total viable organisms was tissue associated in both WT and SP-C-hCFTR+/+ (Fig. 2B) mice, suggesting that the level of CFTR in the lung did not alter bacterial association with the respiratory epithelium. The clearance of this bacterial dose by FABP-hCFTR+/−-mCFTR−/− mice was also not different from WT mice (not shown).

The role of CFTR in bacterial clearance was also determined after an acute (1.5 × 10^7 CFU) intratracheal infection with FRD1 bacteria in WT, heterozygous SP-C-hCFTR+/−, homozygous SP-C-hCFTR+/+, and FABP-hCFTR+/−-mCFTR−/− mice. Bacterial clearance was similar in all mice except that a small but statistically significant increase in viable bacteria (compared with WT mice) was detected at 24 h in homozygous SP-C-hCFTR+/+ mice (Fig. 3). Similar bacterial numbers were observed at 6 h postinfection in all 4 genotypes (not shown). There was no significant difference in bacterial clearance at 24 h between WT and SP-C-hCFTR+/− mice infected with a similar intratracheal dose (1.5 × 10^7 CFU) of the nonmucoid PA01 strain (Fig. 3B). However, PA01 was eliminated more efficiently than FRD1 (compare Fig. 3, A and B) in both WT and transgenic mice, suggesting that mucoidy or other strain-dependent factors may play a role in bacterial survival, but was not influenced by the level of CFTR.

TNF-α and IL-1β in lungs of CFTR transgenic mice after PA infection

To determine whether there is a relationship between CFTR expression levels and bacterially induced inflammation, we measured the concentrations of TNF-α and IL-1β in WT and transgenic mice in lung homogenates or lung lavage from mice infected with FRD1. As shown in Fig. 4, a subacute intratracheal dose of this organism caused a rapid increase in TNF-α production between 2 and 3.5 h postinfection in both WT and SP-C-hCFTR+/− mice. The levels of TNF-α declined rapidly between 6 and 24 h postinfection. Greater than 75% of TNF-α was recovered in lung lavage from both mouse groups (Fig. 4C) consistent with a rapid secretion of this cytokine in the alveolar space. A statistically significant increase in TNF-α was observed in SP-C-hCFTR+/+ and FABP-hCFTR+/−-mCFTR−/− mice compared with WT mice 24 h after an acute intratracheal dose of FRD1 (Fig. 5A). This was not the case in lung homogenates of WT and SP-C-hCFTR+/+ mice assessed 24 h after a similar infectious dose of PA01 (Fig. 6A).

Similar levels and kinetics of IL-1β protein were observed in lung tissue and lavage from WT and SP-C-hCFTR+/− mice (Fig. 7) after intratracheal administration of FRD1. The level of IL-1β in lung tissue peaked at 3.5 h (Fig. 7B) and declined gradually by 50% at 24 h postinfection in both mouse groups, whereas the amount of secreted IL-1β recovered in lung lavage remained constant after 6 h (Fig. 7C). Between 1 and 10% of IL-1β was secreted in the alveolar compartment over the 24-h period, indicating that only a small fraction of this cytokine is processed to the mature form and secreted into the alveolus (compare Fig. 7, C to A). Similar amounts of IL-1β were also detected in WT and FABP-hCFTR+/−-mCFTR−/− mice 24 h after an acute intratracheal dose of FRD1 (Fig. 5B). However, the amount of IL-1β in SP-C-hCFTR+/+ mice was significantly higher than in WT and FABP-hCFTR+/−-mCFTR−/− mice 24 h after infection with FRD1 (Fig. 5B). In contrast to FRD1, similar IL-1β levels were observed after an acute infection of SP-C-hCFTR+/− mice with PA01 bacteria (Fig. 6B). Cressman et al. (10) also observed similar clearance of PA01 between mCFTR−/− mice and different strains of WT mice.

The time course of TNF-α and IL-1β production in WT and SP-C-hCFTR+/− mice in response to the acute infective dose of FRD1 is shown in Fig. 8. A sustained production of TNF-α was measured in both WT and SP-C-hCFTR+/− mice with significantly higher levels of TNF-α being observed in SP-C-hCFTR+/+ mice at 6 and 24 h postinfection (Fig. 8A). The concentration of IL-1β in the lung also remained constant in WT mice over the 24-h infection period (Fig. 8B). However, the level of IL-1β in SP-C-hCFTR+/+ mice was significantly higher than WT and continued to increase over the 24-h infection period.

The neutrophil influx in the lungs of WT and CFTR transgenic mice was monitored in BAL after a subacute infective dose of 3 × 10^6 FRD1. The time-course and extent of neutrophil recruitment was similar in both WT and the various CFTR transgenic mice (data not shown). At 3.5 h postinfection the mean percent neutrophil content was 4.8 ± 1.6, 29.9 ± 4.2, 57.2 ± 7.9, and 60.9 ± 5.1 in sham WT controls and infected WT, SP-C-hCFTR+/+, and
Bacterial clearance after an acute pulmonary infection with PA FRD1 and PA01. A, Eight-week-old WT (□, n = 26), heterozygote SP-C-hCFTR\(^{+/−}\) (●, n = 8), homozygote SP-C-hCFTR\(^{+/+}\) (■, n = 15, p = 0.044 compared with WT mice), and FABP-hCFTR\(^{+/−}\)-mCFTR\(^{+/−}\) (○, n = 11) mice were infected intratracheally with 1.5 × 10\(^5\) CFU of FRD1. Bacterial CFU were determined in tissue homogenates at 24 h postinfection as described in Materials and Methods. Error bars are means ± SE. All p values comparing statistical differences between WT and SP-C-hCFTR\(^{+/−}\) and FABP-hCFTR\(^{+/−}\)-mCFTR\(^{+/−}\) mice were >0.05. B, Eight-week-old WT (□, n = 26) and homozygote SP-C-hCFTR\(^{+/+}\) (■, n = 8) were infected intratracheally with 1.5 × 10\(^5\) CFU of strain PA01. Bacterial CFU were determined in tissue homogenates at 24 h postinfection as described in Materials and Methods. Error bars are means ± SE. All p values comparing statistical differences between WT and SP-C-hCFTR\(^{+/+}\) were >0.05.

Discussion

A number of studies indicate that CFTR influences both humoral and cell-mediated pathways of innate immunity in the lung (9, 11). We evaluated the pathology of PA pneumonia by histology to determine whether CFTR expression in the homozygous mice influences both humoral and cell-mediated pathways of innate immunity in the lung (9, 11). A diffuse alveolitis with marked congestion of the pulmonary capillaries was observed in both WT and SP-C-hCFTR\(^{+/−}\) mice (Fig. 9). Increased numbers of bacteria with the formation of microabscesses and evidence of microhemorrhages were found in the more severely infected SP-C-hCFTR\(^{+/−}\) mice (Fig. 9B). In both WT (data not shown) and SP-C-hCFTR\(^{+/−}\) (Fig. 10, A and D) mice a large number of bacteria had invaded interstitial spaces beneath bronchiolar and alveolar epithelia. Sites of epithelial erosion and denudation were observed across bronchiolar epithelia in the lungs of both WT (data not shown) and SP-C-hCFTR\(^{+/−}\) mice (Fig. 10B), and clusters of bacteria were associated with membranous exudates in the airways of both WT and SP-C-hCFTR\(^{+/−}\) mice (Fig. 10B). In both WT and SP-C-hCFTR\(^{+/−}\) mice, neutrophils were associated with high concentrations of bacteria. Despite the large number of organisms in the airways of both WT and SP-C-hCFTR\(^{+/−}\) mice, bacteria were found in neutrophils of both mice but not in the alveolar type II or bronchiolar epithelial cells where the hCFTR transgene is expressed (41). Furthermore, even though increased bacteria were observed in SP-C-hCFTR\(^{+/−}\) mice (Fig. 2), the distinctive pathological features of PA pneumonia were similar in both WT and SP-C-hCFTR\(^{+/−}\) mice.

PA pneumonia in the lungs of WT and SP-C-hCFTR\(^{+/+}\) mice

We evaluated the pathology of PA pneumonia by histology to determine whether CFTR expression in the homozygous mice influenced bacterial dissemination into epithelial cells and, perhaps, bacterial virulence. The histopathology of PA infection in WT and SP-C-hCFTR\(^{+/+}\) mice 24 h after an acute pulmonary infection with strain FRD1 is shown in Figs. 9 and 10. A diffuse alveolitis with marked congestion of the pulmonary capillaries was observed in both WT and SP-C-hCFTR\(^{+/+}\) mice (Fig. 9). Increased numbers of bacteria with the formation of microabscesses and evidence of microhemorrhages were found in the more severely infected SP-C-hCFTR\(^{+/+}\) mice (Fig. 9B). In both WT (data not shown) and SP-C-hCFTR\(^{+/+}\) (Fig. 10, A and D) mice a large number of bacteria had invaded interstitial spaces beneath bronchiolar and alveolar epithelia. Sites of epithelial erosion and denudation were observed across bronchiolar epithelia in the lungs of both WT (data not shown) and SP-C-hCFTR\(^{+/+}\) mice (Fig. 10B), and clusters of bacteria were associated with membranous exudates in the airways of both WT and SP-C-hCFTR\(^{+/+}\) mice (Fig. 10B). In both WT and SP-C-hCFTR\(^{+/+}\) mice, neutrophils were associated with high concentrations of bacteria. Despite the large number of organisms in the airways of both WT and SP-C-hCFTR\(^{+/+}\) mice, bacteria were found in neutrophils of both mice but not in the alveolar type II or bronchiolar epithelial cells where the hCFTR transgene is expressed (41). Furthermore, even though increased bacteria were observed in SP-C-hCFTR\(^{+/+}\) mice (Fig. 2), the distinctive pathological features of PA pneumonia were similar in both WT and SP-C-hCFTR\(^{+/+}\) mice.

Discussion

A number of studies indicate that CFTR influences both humoral and cell-mediated pathways of innate immunity in the lung (9,
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FIGURE 5. Cytokine levels after an acute pulmonary infection with PA FRD1. Eight-week-old WT (▲, n = 26) and homozygote SP-C-hCFTR^{+/+} (■) mice, and 8- to 10-wk-old FABP-hCFTR^{+/−}·mCFTR^{−/−} (□) mice were infected intratracheally with 1.5 × 10^7 CFU of strain FRD1. Cytokine levels were determined in lung tissue homogenates as described in Materials and Methods. A, Production of TNF-α was measured by ELISA (R&D) in lung homogenates from control (n = 26), SP-C-hCFTR^{+/+} (n = 15, p = 0.004 compared with WT), and FABP-hCFTR^{+/−}·CFTR^{−/−} (n = 11, p = 0.04 compared with WT). B, Production of IL-1β was measured by ELISA (R&D) in lung homogenates from control (n = 26), SP-C-hCFTR^{+/+} (n = 15, p = 0.002 compared with WT and p = 0.018 compared with FABP-hCFTR^{+/−}·mCFTR^{−/−}), and FABP-hCFTR^{+/−}·mCFTR^{−/−} (n = 11, p = 0.072 compared with WT and p = 0.1158 compared with SP-C-hCFTR^{+/+}) mice. Error bars are means ± SE.

11–13, 20–25). In this report we have used WT and CFTR transgenic mouse lines to test whether CFTR influences the clearance of PA by lung epithelial cells in vivo. Recent studies suggested that the extracellular CFTR sequence at amino acids 103–117 binds the core oligosaccharide component of LPS on the surface of PA leading to the internalization of the bacterium by epithelial cells (31–34). It remains unclear whether these interactions influence clearance and inflammation following PA infection in vivo. In this study, mice were infected with both mucoid PA strain FRD1 and nonmucoid strain PA01 (43, 44). Mucoid PA strains lack the O-Ag side chains but retain the core oligosaccharide structure, the presumptive ligand for CFTR (47). We also used the nonmucoid PA01 strain, a widely used laboratory strain that originated from a wound infection. Strain PA01 was included in earlier studies concerning the role of CFTR in the uptake of PA by epithelial cells (31–34). This strain is representative of the “smooth” PA strains that infect the CF host early in the course of the disease, whereas strain FRD1 represents the “rough” LPS variant that predominates during the course of the disease. These findings, demonstrating similar clearance of PA in WT, heterozygote SP-C-hCFTR^{+/−}, and FABP-hCFTR^{+/−}·mCFTR^{−/−} mice expressing normal, increased, or no CFTR in the lung, respectively, do not support a direct relationship between the level of CFTR and acute bacterial clearance from the adult mouse lung. Furthermore, the small but significant proliferation of bacteria in the lungs of homozygous SP-C-hCFTR^{+/+} mice does not correlate with a proposed beneficial role of CFTR in PA clearance.

The absence of differences in clearance of PA between WT and transgenic mice expressing different levels of CFTR suggests that, among multiple humoral and cell-mediated clearance mechanisms in the lung, a direct CFTR-dependent mechanism is not an important component of PA clearance in vivo model. Previous studies in mouse models indicate that neutrophils are a critical component in the clearance of PA. We measured bacterial numbers in lavage and postlavage tissue homogenates from WT and CFTR-overexpressing mice at 2 h postinfection with strain FRD1, before a significant neutrophil influx and at later time points, and found no significant differences in tissue-associated bacteria. Similar numbers of tissue-associated bacteria were observed in both groups of mice, suggesting the retention of PA that was independent of CFTR. Furthermore, WT and FABP-hCFTR^{+/−}·mCFTR^{−/−} mice had similar bacterial numbers after infection with an acute dose of PA strain FRD1. Similar infection levels were also observed in WT and SP-C-hCFTR^{+/+} mice 24 h after an acute dose of PA.
strain PA01. These findings support previous studies that reported a lack of chronic PA infection with PAO1 in CFTR knockout mice with allergic inflammation (10) and efficient PA killing in transgenic mice expressing CFTR carrying the ΔF508 mutation (9).

The homozygous SP-C-hCFTR

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mice had a small but significant increase in bacterial burden 24 h after infection (Fig. 2) with the mucoid PA strain FRD1, suggesting a negative rather than a protective role of higher CFTR levels in recovery from an acute bacterial infection. Lung pathology following acute infection with FRD1 was similar in WT and SP-C-hCFTR

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mice (Figs. 10, 11, and 12), and there was no evidence of selective uptake or association of bacteria in alveolar type II or bronchiolar epithelial cells, the cell sites of SP-C-hCFTR

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transgene expression (41). These results indicate that CFTR expression does not correlate with bacterial attachment, uptake, or clearance by respiratory epithelial cells in the SP-C-hCFTR

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mouse model.

The prominent histopathological characteristics of PA-induced pneumonia seen in the mouse lung are consistent with the lung disease that PA induces in hospital-acquired pneumonia in humans and in rat models of PA infection (47, 48), and is distinct from chronic infection seen in CF or in agar bead models of infection (11–14). The distinctive bacterial penetration into interstitial sites and localization at basal surfaces of the bronchiolar epithelia are consistent with previous in vitro studies with nonmucoid strains that implicated PA virulence with access and binding of PA to the basolateral epithelial membrane (36, 37, 39, 49–51). Invasion of PA bacteria to the basolateral surface in vitro is thought to occur through weakened tight junctions during injury-induced epithelial repair or at free epithelial edges in tissue culture epithelia (37). In this in vivo study, we found patches of bronchiolar epithelial cell erosion that could provide entry sites for bacterial invasion into basolateral and interstitial compartments. The mechanisms that underlie the more severe pneumonia in homozygote transgenic mice (Figs. 2, 9, and 10) are not presently known. However, Rezaiguia et al. (52) demonstrated that acute pneumonia with PA in rats increases fluid clearance from the lung, a process that is associated with acute lung injury. Additional studies should determine whether CFTR has a role in the coordination of fluid dynamics and host-pathogen interactions during PA pneumonia.

The production of TNF-α and IL-1β was assessed in WT and various CFTR transgenic mice to determine whether the level of CFTR expression influenced the evolution of pulmonary inflammation in response to PA infection. A similar transient increase in

FIGURE 7. Kinetics of IL-1β synthesis after a subacute pulmonary infection with PA FRD1. Eight-week-old littermate control [□] and heterozygote SP-C-hCFTR

+/−

[■] mice were infected intratracheally with 3 × 10^6 CFU of strain FRD1. The levels of IL-1β were measured by ELISA in alveolar lavage and postlavage tissue homogenates at 2 (n = 5), 3.5 (n = 10), 6 (n = 4), and 24 h (n = 5) postinfection as described in Materials and Methods. Upper, Sum of IL-1β levels in lavage and postlavage tissue homogenate, representing an estimate of IL-1β in whole lung. Middle, Total IL-1β present in postlavage tissue homogenate. Lower, Total IL-1β present in alveolar lavage. Error bars are means ± SE. All p values comparing statistical differences between WT and SP-C-hCFTR

+/−

were >0.05.

FIGURE 8. Kinetics of cytokine synthesis after an acute pulmonary infection with PA FRD1. Eight-week-old WT [□] and SP-C-hCFTR

+/−

[○] mice were infected intratracheally with 1.5 × 10^7 CFU of strain FRD1. The levels of TNF-α were measured by ELISA in tissue homogenates at 6 and 24 h postinfection as described in Materials and Methods. A, TNF-α levels in tissue homogenate at 6 (n = 12, p = 0.0087) and 24 h (n = 15, p = 0.005) postinfection. B, IL-1β levels present in tissue homogenate at 6 (n = 12, p = 0.017) and 24 h (n = 15, p = 0.002) postinfection. Error bars are means ± SE.
A diffuse alveolitis consisting primarily of neutrophils was observed in WT mice. The capillary bed was also congested, and clusters of bacteria and membranous exudates were found in the alveoli. Increased numbers of bacteria with microabscesses and focal areas of microhemorrhages (arrows) were observed in the SP-C-hCFTR \textsuperscript{+/−} mice. Sections were stained with H&E. Original magnification for A and B = ×460. Bar = 100 μm.

Histopathology of PA infection in WT and SP-C-hCFTR \textsuperscript{+/−} mice 24 h after an acute pulmonary infection with strain FRD1. A, Invasion of peribronchiolar interstitial regions with bacteria. Note association of bacteria with the basal aspect of the bronchiolar epithelium (arrowheads). B, Bacterial invasion with colonization of eroded bronchiolar epithelium was also found (arrow). C, Clusters of neutrophils with adherent bacteria were found throughout the parenchyma (arrowheads). Note the lack of association of bacteria with type II cells in this region (arrows). Sections were stained with Giemsa. Original magnification for A–C = ×920. Bar for A–C = 50 μm. Original magnification for D–F = ×1380. Bar for D–F = 30 μm.
higher cytokine expression observed in FABP-hCFTR"-
+/- and SP-C-hCFTR"+- with FRD1 infection was not observed following an acute infection with the nonmucoid PA01 strain; however, PA01 was cleared more efficiently than FRD1, suggesting a potential complex relationship between CFTR and inflammatory responses to different bacterial infections in vivo.

The results of this study do not support a direct role of CFTR in bacterial clearance after an acute PA infection but may implicate a role for CFTR expression in pulmonary inflammatory responses during acute bacterial infections in transgenic mice. Our findings do not support a model in which PA binds to CFTR to influence bacterial clearance as seen in a neonatal mouse model of acute PA infection (33). Histologic findings in this study support the inva-
sive nature of acute PA pneumonia but do not support a direct association of PA with bronchial or alveolar type II epithelial cells, or a clear correlation between varying CFTR expression and bacterial clearance in adult mice. The relationship between the level of CFTR expression and excessive inflammatory responses merits further investigation.

Acknowledgements

We thank Paola Blair for technical support with tissue processing for histo-

References


