G551D CF mice display an abnormal host response and have impaired clearance of Pseudomonas lung disease

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Cystic fibrosis (CF) is most commonly characterized by chronic bacterial infections of the upper respiratory tract, leading to intense inflammation, lung damage, respiratory failure, and eventual death. The relationship between the biochemical defect in CF, a lack of CF transmembrane conductance regulator (CFTR)-mediated chloride secretion in epithelial cells, and susceptibility to bacterial infection has yet to be determined (6). Most patients acquire chronic infections of Pseudomonas aeruginosa that are associated with a significant decline in pulmonary function and are the leading cause of morbidity and mortality (12, 34). Predisposition to this relatively benign environmental organism is believed to result from several CF-specific host factors that alter host-pathogen interactions. Airway secretions in CF are highly viscous and may impede mucociliary clearance of bacteria (2, 17). Such secretions are also predicted to have increased concentrations of NaCl that inactivate epithelial cell-derived antimicrobial proteins called β-defensins (9, 35). Functional CFTR can specifically mediate engulfment of P. aeruginosa by epithelial cells, which are subsequently shed by cell sloughing (25–27). CF epithelial cells may also have an increased number of cell surface receptors for P. aeruginosa (14, 31, 40).

Apart from the effect of the CF mutation on epithelial-bacterial interactions, the CF lung also appears to mount an excessive but ineffective response to invading microbes. Infected airways are dominated by neutrophil infiltration and high levels of proinflammatory mediators, proteases, and oxygen radicals that damage epithelial tissues and fail to clear the bacteria. Patients with only mild pulmonary symptoms show signs of inflammation, and CF infants with no detectable infections have elevated levels of proinflammatory cytokines (15, 16, 22, 23). Experiments using cultured primary tissues and immortalized cell lines indicate host-response defects in epithelial cells, including excessive production of interleukin-8 and increased amounts of active nuclear factor-κB, a transcriptional activator central to proinflammatory gene regulation (1, 37, 38, 43). CFTR mutations have also been reported to have cell-autonomous effects in immune cells (21, 41).

To better understand the pathogenesis of lung disease in CF, mouse models with defective murine CFTR have been created (4, 7, 24, 28, 42), and several have been subjected to pulmonary bacterial challenge (5, 11, 13, 18, 36). Although most of these studies suggest that CF mice are more susceptible to lung infection, none of the models is entirely satisfactory. Mice are relatively resistant to lung disease, and the method of challenge can affect the outcome significantly. Methods that most accurately mimic the chronic infection in human CF patients utilize mucoid P. aeruginosa entrapped in small (150-μm-diameter) agar beads. Mucoid strains of this bacteria are most commonly associated with chronically infected CF lungs, and the beads mechanically localize the bacteria in the lower airways and...
mimic the microcolony morphology observed in the CF lung (10, 13). Intratracheal inoculation of the lung with such beads allows lung infections to persist for at least 28 days in non-CF mice (32, 39). CF mice appear more susceptible to bead challenge, with high incidences of mortality and signs of severe inflammatory disease recorded. In a CFTR knockout mouse strain, abnormally high levels of the proinflammatory mediators tumor necrosis factor (TNF)-α, KC/N51 (KC), and macrophage inflammatory protein (MIP)-2 were observed 3 days after exposure to Pseudomonas agar beads, and almost 80% of these animals died after 7 days (13). When the same CFTR knockout was present on an inbred genetic background (C57BL/6), the mice showed an impaired ability to clear P. aeruginosa agar beads between 3 and 6 days after infection compared with wild-type mice, and mortality rates were >50% (11). These data strongly support the notion that mice lacking CFTR function are predisposed to bacterial lung infection and are therefore a relevant model in which to study the disease. Although the mechanism of increased susceptibility has yet to be determined in CF mice, it is hypothesized that the underlying CF-specific host factors are the same as in humans.

Delaney et al. (7) have previously generated a novel CF mouse homozygous for the human CF allele G551D, which lacks CFTR function and displays a relatively mild gut pathology. The G551D strain also displays an innate defect in the immunologic response to inflammatory stimuli (41). We observed excessive levels of S100A6 protein, a marker of neutrophil infiltration, and higher than normal numbers of neutrophils in the lungs of G551D mice after intravenous challenge with bacterial lipopolysaccharide (LPS) as well as a hyper-sensitive macrophage phenotype. Here we describe the effects of the P. aeruginosa agar bead model of endobronchial infection on G551D CF mice. In contrast to other CF mouse studies, we measured significantly reduced rates of pulmonary clearance of bacteria in the G551D animals, suggesting that this CF mouse may more closely model CF lung disease. Measurement of inflammatory cytokines during the infection revealed a paradox that was not observed previously: initial hyperresponsiveness was followed by almost complete suppression of the response. We speculate that the initial hyperinflammatory response in CF patients actually compromises an effective defense against P. aeruginosa in the CF lung.

METHODS

Animal resources. This study utilized the CF mouse model strain carrying the G551D mutation in the CFTR gene (7). The genetic background of the mice is a cross of the CD1 and 129sv strains. The animals were genotyped with tail DNA PCR as previously described (7). G551D mice and control littermates were housed together under identical specific pathogen-free conditions before experimentation. The pups were weaned and fed a liquid elemental diet (Peptamen, Nestle, Brisbane, Australia) ad libitum beginning at 14 days of life to avoid the intestinal obstruction problems that can occur in this strain of mouse (7). Most G551D animals reached adulthood, had grossly normal lungs, and when fully grown weighed 25.1 ± 0.60 g compared with 29.9 ± 0.55 g for their non-CF littermates. Animals were used for experimentation between the ages of 8 and 15 wk.

All animal experimentation was conducted in accordance with National Health and Medical Research Council of Australia guidelines and was approved by the University of Queensland Animal Ethics Committee.

Infection of mice. A chronic P. aeruginosa endobronchial infection was established in the mice with the agar bead method described by Gosselin et al. (10) with several modifications. Briefly, a log phase culture of P. aeruginosa strain M57-15, a mucoid clinical isolate (13), was diluted into warm (52°C) trypticase soy agar (BBL, Cockeysville, MD). The bacteria were then entrapped in agar beads by mixing with heavy mineral oil (20% to 30%) and then homogenized in 50-ml sterile PBS. The beads were passively filtered through sterile nylon mesh (200-μm pore diameter) and then entrapped in agar beads and then then cultured on isolation agar (PIA; Difco, Sparks, MD) supplemented with 5% fetal calf serum (BioWhittaker, Walkersville, MD) and then subjected to quantitative bacteriology as described in Recovery of BAL fluid, Quantitative bacteriology, and Histopathological studies. Each CF mouse was matched with a G551D heterozygous or wild-type littermate as a non-CF control. The total number of mice infected and killed on each day was consistent proportion of inoculum was delivered to the lungs. One heterozygous and/or wild-type littermate as a non-CF control. The total number of mice infected and killed on each day was consistent proportion of inoculum was delivered to the lungs.
analysis. BAL was performed by cannulating the trachea in situ with a blunt-end 21-gauge needle, instilling 1.0 ml of sterile PBS three times, and collecting the fluid by gentle aspiration. The total BAL fluid (BALF) recovered was 2.3–2.8 ml. The fluid was centrifuged for 10 min, and the supernatants were stored at −80°C after the addition of EDTA and phenylmethylsulfonyl fluoride to 5 and 0.1 mM, respectively. The cell pellet was resuspended in 0.5 ml of PBS, 0.1% BSA (ICN Biomedicals, Aurora, OH), and 0.1% sodium azide (PBA buffer) and used immediately for flow cytometry analysis as described in Analysis of mouse BALF cells with flow cytometry.

Quantitative bacteriology. After BAL, the lungs and trachea were aseptically dissected and homogenized in 3 ml of sterile PBS. Samples of lung and BALF homogenates were serially diluted and cultured on PIA (Difco) supplemented with 5% fetal calf serum (BioWhittaker). Mucoid colonies of P. aeruginosa were visualized after incubation for 24 h at 37°C. The spleens were removed and homogenized in 1.0 ml of PBS, and a 50-μl aliquot was cultured on PIA to test for systemic P. aeruginosa infection. Positive cultures were observed in 5 infected animals (3 CF and 2 heterozygotes).

Analysis of mouse BALF for proinflammatory cytokines. Murine TNF-α, murine MIP-2, and murine KC were measured with commercially available enzyme-linked immunosassays according to the manufacturer’s recommended protocols (R&D Systems, Minneapolis, MN). The limit of detection for the cytokines was 5.0 pg/ml. The BALF supernatant and serum samples were assayed in duplicate. The BALF values were corrected for the respiratory epithelial lining fluid volume recovered by measuring the urea dilution (29).

Analysis of mouse BALF cells with flow cytometry. Leukocyte populations in the BALF were differentiated and quantified with antibody staining and flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) against mouse CD45 (clone YW62.3, rat IgG2b), CD3 (clone KT3, rat IgG2a), macrophages (clone MOMA-2, rat IgG2b), and Ly6G/Gr-1 (clone RB6–8C5, rat IgG2b) were used. In each experiment, FITC-labeled anti-rat IgG anti-IgG2b, and Ly6G/Gr-1 (clone KT3, rat IgG2a), macrophages (clone MOMA-2, rat IgG2b) were used. In each experiment, FITC-labeled anti-rat IgG antibody was used as a negative control. Anti-Ly6G MAb was purchased from Leinco Technologies (St. Louis, MO) and all other MAbs were obtained from Serotec (Raleigh, NC). Suspended BALF cells were fixed in 1% paraformaldehyde (PFA) for 30 min at 4°C and then washed with 10 ml of PBA, centrifuged for 4 min at 1,000 g, and resuspended in 0.5 ml of PBA. Aliquots (60 μl) of cells were blocked by addition of 6 μl of 1% goat serum (BioWhittaker) for 15 min followed by 30 min of incubation with individual FITC-labeled MAbs (5 μl). An additional 60-μl aliquot was preincubated in 0.2% Tween 20 for 15 min at 37°C to permeabilize the cells before they were blocked and stained with MOMA2 MAb, which is an intracellular antigen. Stained cells were washed (10 ml), resuspended in PBA (0.4 ml), and immediately subjected to flow cytometry with FACScan II (Becton Dickinson, Mountain View, CA). Acquisition and analysis of the data were performed with CellQuest software (Becton Dickinson). For each sample, 10,000 events were collected and the number of positive cells was determined. The total number of cells per lung was calculated using the time taken to collect 10,000 cells, the appropriate sample dilution, and the correction for the respiratory epithelial lining fluid volume recovered by measuring the urea dilution (29).

Histopathological studies. The lungs of infected mice not subjected to BAL were inflation fixed in 4% PFA in PBS for 36 h followed by incubation in progressively increasing concentrations of optimum cutting temperature compound (Sakura Finetek USA, Torrance, CA) in 15% sucrose and PBS and finally frozen in optimum cutting temperature compound. Longitudinal sections (12 μm) were taken at regular intervals from the ventral to dorsal surface. The sections were stained with hematoxylin and eosin with standard techniques and quantified for pathological involvement with a blind grid-square analysis as follows. Color photographs of stained whole lung sections (4 sections/animal) were overlaid with a 1-cm² grid, and the absence or presence of inflammation (evidenced by lightly stained, healthy tissue or darkly stained, cell-dense tissue, respectively) was scored at each intersecting line on the grid. At least 120 individual scores were made on each section, and the investigator was not aware of the genotype of each section during the analysis. The number of mice analyzed was 2 CF and 3 non-CF on day 1 and 5 CF and 11 non-CF on day 3.

Statistical analysis. Data are expressed as means ± SE. Data comparing bacterial burden in the lungs of non-CF and G551D mice were analyzed with the nonparametric Mann-Whitney U-test. Weight loss, concentrations of inflammatory cells and proinflammatory cytokines, and histopathological involvement were compared with an unpaired, two-tailed Student's t-test. The relationships between the infected animals’ weight loss, bacterial burden, and inflammatory response were assessed by the Pearson correlation coefficient.

RESULTS

G551D mice exhibit excessive weight loss after infection. To establish a model of chronic infection, cohorts of G551D homozygous (CF), heterozygous, and wild-type littermates were given endobronchial infections of mucoïd P. aeruginosa entrapped in agar beads and monitored for 1, 2, or 3 days. For this study, the genetic background is a complex mixture resulting from the outbreeding of the original knockout allele in 129Sv with CD1 mice. The effect of the genetic background is partly controlled by the use of littermates from multiple independent litters. However, we also feel that the use of outbred rather than inbred strains, which themselves have idiosyncratic responses to infection with this organism, provides a greater level of confidence that the results are relevant to disease in a genetically diverse human population.

Infected animals displayed visible responses to the challenge during the first 24 h, including lethargy, piloerect coats, and breathing difficulties. These symptoms persisted in the CF animals in the following 2 days but appeared to resolve in most heterozygous and wild-type littermates. In essence, the outbred mice were generally resistant to this infection as are normal humans. Two CF mice and one heterozygous mouse died ~48 h after infection. Bacteria were present in the spleens as well as the lungs of these mice, suggesting that death was due to bacteremia.

The animals were weighed daily, and significant weight losses were observed throughout the 3-day period (Fig. 1). On average, the heterozygous and wild-type animals lost around 5% of their body weight each day after infection. By contrast, the CF mice lost over twice as high a proportion of their body weight on the first day. The difference compared with control littermates was highly significant (P < 0.001). Thereafter, the CF mice continued to lose weight in parallel with their littermates.
G551D mice are unable to clear bacterial lung infections. To compare how successful mice were in controlling and clearing the infection, the lungs were excised from groups of animals 1, 2, or 3 days after infection, and quantitative bacterial count assays were performed (Fig. 2). After 24 h, the number of bacteria recovered was consistently 10-fold greater than the initial dose, indicating that they were viable and proliferating. Significant variation was observed in the burdens from mice 2 and 3 days after bead inoculation, suggesting a variable ability to control the infection. The majority of heterozygous and wild-type animals killed on day 3 (18 of 29) contained fewer bacteria than the inoculum dose (ranging from 50,000 to 90,000 CFU), whereas in most CF animals (17 of 19), the burden was far greater. No differences in clearance were observed between the heterozygous and wild-type animals, but a significant difference was found between CF and non-CF mice infected for 3 days (log average bacteria 6.60 and 4.95, respectively; \( P < 0.002 \)). In pairwise comparisons between individual CF and non-CF littermates that survived for 3 days, 17 of 19 CF animals harbored more bacteria (\( P = 0.012 \)). There were positive correlations between weight loss and bacterial burden in both CF and non-CF mice.
examined 3 days after infection (CF: \( r = 0.611, P = 0.005 \); non-CF: \( r = 0.409, P = 0.004 \)), suggesting that infection contributed to weight loss. Although the average initial weight of the CF group was 16% less than that of the non-CF mice, no correlation between weight and clearance was found. In addition, there were four cases where pairs of CF and non-CF littermates had similar weights, but the CF animals still had higher bacterial burdens. Thus the smaller size of the G551D mice was unlikely to be the reason for their increased susceptibility.

**BALF cytokine profiles differ between G551D and non-CF mice.** The proinflammatory cytokines TNF-\( \alpha \), KC, and MIP-2 are produced by the murine lung in response to infection and play several important roles in host defense. BALF from infected CF and non-CF mice contained significant levels of all three molecules (Fig. 3). Non-CF animals produced relatively high and sustained levels of TNF-\( \alpha \) in the 3 days after infection. In contrast, TNF-\( \alpha \) concentrations in the CF mice showed a significantly different pattern of expression, being higher 1 day postinfection but declining rapidly thereafter. Significant levels of KC and MIP-2 were observed in non-CF mice during the first 2 days and lower amounts by the third day. Production of these molecules in CF mice was slightly higher at 24 h but was again reduced markedly by day 2. Levels of KC in the G551D mice were significantly less than non-CF concentrations on day 2, whereas MIP-2 levels were also lower but did not reach significance (\( P = 0.07 \)). Although no differences were observed between non-CF and CF mouse cytokine levels on day 3, positive correlations were observed collectively between bacterial burden in the lung and levels of TNF-\( \alpha \) (\( r = 0.306; P = 0.03 \)) and MIP-2 (\( r = 0.463; P = 0.007 \)), suggesting that levels of these molecules are associated with the outcome of the infection.

**Increased inflammation in infected G551D mice.** Histopathological examination of the lungs of infected animals revealed signs of significant inflammatory lung disease (Fig. 4). In most sections from 1-day infected mice, we observed many medium and small
airways obstructed with beads and localized cell inflammation (Fig. 4, A and C). In contrast to the marked difference in LPS-induced neutrophil infiltration previously reported by Thomas et al. (41), there was no obvious difference between non-CF and CF animals. After exposure to bacteria for 3 days, cellular inflammation was more widespread (Fig. 4, B and D). Dense inflammatory cell infiltrate was observed within infected bronchi and surrounding tissues of both CF and non-CF mice. Beads were still visible in some sections, suggesting that the infection was sustained. Although the number of mice examined at each time point was limited, a significantly higher proportion of inflammatory involvement was observed in the lungs of 3-day infected CF animals (22.2 ± 2.7%; n = 5) compared with similarly infected non-CF mice (15.9 ± 2.4%; P < 0.01; n = 11).

We also identified and quantified the types of leukocytes recovered from the BALF (Fig. 5). Dramatic increases in leukocyte (CD45-positive) populations were observed after infection. This increase was attributed mostly to neutrophils (Gr-1), which comprised over 90% of CD45-positive cells. There were no differences between CF and non-CF neutrophil levels, but in the non-CF group, the number on day 3 was significantly less than that on day 2, suggesting that the inflammatory response was abating. Macrophage (MOMA2) and CD3-positive T-cell populations were also detected as minor components, and the levels did not change appreciably after infection. Together, the data suggest that in terms of inflammatory cell involvement, airway inflammation appears equivalent between CF and non-CF animals. However, given the histological differences, which take into account the entire lung, inflammation of extrabronchial tissues may be increased in the CF mice. These differences at later stages in the inflammatory process may simply be due to the failure of the CF mice to clear the infection.

**DISCUSSION**

CF airways are particularly susceptible to chronic bacterial infection and intense inflammation that results in significant tissue damage and eventual failure of lung function (34). Many studies (1, 9, 14, 16, 17, 21, 25, 37, 38, 41, 43) indicate that CFTR-dependent aberrations in lung homeostasis are central to pulmonary susceptibility. However, elucidation of the underlying mechanisms has been limited by the lack of an appropriate model for CF lung disease. Several studies (5, 18, 36) have described the use of CFTR knockout mice, which, despite being relatively resistant to lung infection, do display some hallmarks of CF lung disease when challenged with aerosolized bacteria. More relevant to CF, chronic models of lung infection have been established in mice with mucoid strains of *P. aeruginosa* entrapped in agar beads (10, 20, 39). Application of this model to CFTR knockout mice results in high rates of mortality and excessive inflammation, indicating that the CFTR-related susceptibility to lung disease can be replicated in mice (11, 13).

Delaney et al. (7) have established a model of CF lung disease using the *P. aeruginosa* agar bead model of endobronchial infection in mice homozygous for the CFTR mutation G551D. This unique animal model of CF replicates a naturally occurring human allele, and unlike the other CF mice that are CFTR null (5, 36), the G551D mutation results in a mature and correctly localized but inactive protein. The most striking feature of this model and of the clinical relevance to lung disease in CF patients was the inability of G551D CF mice to control *P. aeruginosa* lung infection. Bacterial burdens in the CF animals continued to increase during the period of study, whereas the non-CF littermates had a significantly reduced number of bacteria after 3 days. The ongoing weight loss, increasing lung inflammation, and bacterial proliferation also indicated that the CF mice in our study would eventually succumb, whereas the littermates resolve the infection. However, survival data also might be influenced by other effects of the CF gene on nutrition and response to inflammatory cytokines at other sites and are more difficult to interpret than the primary lung pathology.

In the present study, we decided to make a detailed examination of the response of the G551D animals during the early acute phase of infection, between 1 and 3 days after bead inoculation. The outcome of lung disease in chronically infected inbred strains of mice is partly determined by host-response events within 24 h of inoculation, including TNF-α production and inflammatory cell infiltration (10, 19). Therefore, we hypoth-
esized that events during the early establishment of such infections may contribute to the inability of G551D mice to control the bacteria.

Contrary to this expectation, bacterial burden, histopathology, and airway inflammatory cell populations were all remarkably similar between the CF and non-CF mice 24 and 48 h after infection. However, the CF mice responded quite differently in the production of the airway proinflammatory mediators TNF-α, KC, and MIP-2. Concentrations of these molecules, especially TNF-α, were significantly higher than in non-CF littermates 24 h after infection. Perhaps as a direct consequence, given the role of TNF-α in cachexia, the CF mice lost weight twice as rapidly as their littermates in the first 24 h. The surprising finding was that the inflammatory cytokines declined thereafter, so that at 48 h, the effect of the CF gene was the direct opposite. These molecules are important in recruiting inflammatory cells and modulating their bactericidal activities and play protective as well as pathogenic roles in murine lung defense (3, 10, 13, 32). TNF-α functions as a proximal mediator in the inflammatory response and regulates the production of other inflammatory molecules in the lung (8, 33). The magnitude and timing of the TNF-α response is likely to play a central role in the differing susceptibilities of endobronchially infected inbred mice (10). In the G551D mouse, it is therefore possible that an abnormally high initial spike of TNF-α establishes an inappropriate response, but for reasons that are not yet clear, cytokine concentrations are not maintained. There is no clear correlation between the high initial spike in TNF-α and BALF neutrophils or focal inflammation in histological sections, suggesting that the availability of TNF-α and other cytokines locally is not limiting. This finding is not incompatible with the earlier finding by Thomas et al. (41) where intravenous injection of LPS caused a much greater increase in neutrophils in CF than in wild-type mice. We examined the response to LPS at earlier times (90 min and 4 h) when the initial rate of TNF-α secretion may be reflected in the initial rate of neutrophil recruitment. The 24-h time point examined in the bacterial challenge integrates the response over the initial period.

We speculate that it is the decline by 48 h that actually predicts the ineffective bacterial clearance, which could, in turn, result from a failure to activate the inflammatory cells to kill the microorganisms. High TNF-α production during the initial response phase is consistent with previous observations (41) where excessive levels of TNF-α were measured in cultured bone marrow macrophages from G551D animals after LPS stimulation. The diminished response of CF lungs after 48 h might conceivably be a direct consequence of the initial hyperresponsiveness, a kind of tolerance phenomenon, or a feedback loop in which high TNF-α suppresses its own production. Equally, the initial failure to clear or kill the bacteria could lead to suppression by accumulating bacterial products. Determining the cellular and molecular basis of this altered response would greatly improve our understanding of disease progression.

The response to and outcome of chronic Pseudomonas aeruginosa bead infection was different in the G551D animals compared with other CF mouse studies. Heeckeren et al. (13) did not detect any CF-related differences in lung burdens using an outbred CFTR knockout strain and measured an increased inflammatory response 3 days after infection. Significantly higher bacterial burdens were noted in the lungs of similarly infected pure-strain CFTR knockout mice (C57BL/6-CFTR), but unlike the outbred mice used in our study, these mice also suffered from high rates of mortality (11). Survival, clearance of bacteria, and inflammatory responses in mice subjected to endobronchial infection are significantly influenced by the genetic background on which the experiment is performed (20, 39), and genetic factors other than CFTR mutations are likely to have significant influences on the progression of lung disease in CF patients (30). Despite these differences, it is clear that mutations in murine CFTR, whether they deplete or inactivate CFTR, result in heightened susceptibility and aberrant inflammatory responses to chronic lung disease, and these animals should serve as excellent models to dissect the disease pathogenesis. We have recently established the G551D line on resistant and susceptible inbred strains of mice and are currently investigating the contribution of other genetic factors.

Mice carrying inactivating mutations in CFTR are still the only in vivo model available in which to study the causes of CF. We have shown that mice homozygous for the G551D mutation display a CF-like phenotype when challenged with the agar bead model of chronic P. aeruginosa pulmonary infection. Although this model is not representative of the chronic condition per se, indexes relevant to CF, including persistent lung infection, increased weight loss, and an ineffective inflammatory response, indicate its potential use in further understanding the establishment and progression of lung disease and its relationship to defective CFTR function. The phenotype of the G551D mice will also provide a useful and relevant surrogate marker in the development of therapeutics aimed at halting the lung disease.

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