Enhanced Susceptibility to Pulmonary Infection with *Burkholderia cepacia* in *Cftr*⁻⁻ Mice

UMA SAJJAN, GEORGE THANASSOULIS, VERA CHERAPANOY, ANNIE LU, CAROLA SIOLIN, BRENT STEER, YI JUN WU, ORI D. ROTSTEIN, GERALDINE KENT, COLIN MCKERLIE, JANET FORSTNER, AND GREGORY P. DOWNEY

Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Division of Respiratory, Department of Medicine, Climate and Department of Surgery, The University of Toronto, Toronto, Ontario M5S IA8, Comparative Research, Sunnybrook and Women’s College Health Sciences Center, Toronto, Ontario M4N 3M5, and The Toronto General Hospital Research Institute of the University Health Network, Toronto, Ontario M5G 2C4, Canada

Received 5 February 2001/Returned for modification 14 March 2001/Accepted 2 May 2001

Progressive pulmonary infection is the dominant clinical feature of cystic fibrosis (CF), but the molecular basis for this susceptibility remains incompletely understood. To study this problem, we developed a model of chronic pneumonia by repeated instillation of a clinical isolate of *Burkholderia cepacia* (genomovar III, ET12 strain), an opportunistic gram-negative bacterium, from a case of CF into the lungs of *Cftr*⁻⁻ (Cftr⁻⁻) and congenic Cftr⁺⁺ controls. Nine days after the last instillation, the CF transmembrane regulator knockout mice showed persistence of viable bacteria with chronic severe bronchopneumonia while wild-type mice remained healthy. The histopathological changes in the lungs of the susceptible Cftr⁻⁻ mice were characterized by infiltration of a mixed inflammatory-cell population into the peribronchiolar and perivascular spaces, Clara cell hyperplasia, mucus hypersecretion in airways, and exudation into alveolar airspaces by a mixed population of macrophages and neutrophils. An increased proportion of neutrophils was observed in bronchoalveolar lavage fluid from the Cftr⁻⁻ mice, which, despite an increased bacterial load, demonstrated minimal evidence of activation. Alveolar macrophages from Cftr⁻⁻ mice also demonstrated suboptimal activation. These observations suggest that the pulmonary host defenses are compromised in lungs from animals with CF, as manifested by increased susceptibility to bacterial infection and lung injury. This murine model of chronic pneumonia thus reflects, in part, the situation in human patients and may help elucidate the mechanisms leading to defective host defense in CF.

Enhanced Susceptibility to Pulmonary Infection with *Burkholderia cepacia* in *Cftr*⁻⁻ Mice

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the CF transmembrane regulator (CFTR), a transmembrane glycoprotein responsible for chloride conductance in epithelial cells. Progressive pulmonary disease is the dominant clinical feature of CF and accounts for 95% of the morbidity and mortality (11, 51). Despite intensive study, the mechanisms responsible for this enhanced susceptibility to infection remain incompletely understood and a source of controversy. Absence or dysfunction of CFTR leads to alterations in the microenvironment of the lung that are manifest early in life as inflammation of the distal airways (25), but whether this is cause or consequence of infection remains controversial (4). Reported abnormalities in the pulmonary environment in the lungs of animals with CF include altered fluid and ionic fluxes across the respiratory epithelium, excessive luminal mucus, diminished mucociliary clearance, altered patterns of epithelial surface glycosylation, and diminished activity of bacterial factors such as lysozyme, lactoferrin, defensins, and cathelicidins (6, 11, 18, 34, 41, 54, 61). The relative contributions of these factors to airway infection in CF remain unknown.

Whatever the proximate cause of the susceptibility to infection, the milieu of the lungs of animals with CF provides a favorable niche for bacterial infection with certain opportunistic pathogens such as *Staphylococcus aureus* and eventually resistant gram-negative organisms such as *Pseudomonas aeruginosa*. A subgroup consisting mainly of adolescent and adult patients also develop chronic infection with resistant gram-negative bacterium *Burkholderia cepacia*. The clinical outcome after acquisition of *B. cepacia* is variable, ranging from an asymptomatic culture-positive state to a devastating syndrome of fatal necrotizing pneumonia and septicemia (cepacia syndrome) (12, 16, 60). Why CF predisposes patients to acquisition of *B. cepacia* is incompletely understood. Host lung factors such as underlying lung damage (16, 23), repeated exposure (15, 28, 37, 53), and specific bacterial factors, such as presence of cable pili (46–48), production of extracellular enzymes (30, 39), and the ability of some strains of *B. cepacia* to replicate intracellularly (3, 33, 43), all appear to contribute to the propensity to persistent infection.

One feature that has thwarted the identification of virulence properties is that *B. cepacia* is not a single clonal strain. Rather, it consists of a complex of strains that belong to one of five or more genetic groups or genomovars (2, 62). The most common groups cultured from sputa from CF patients are genomovars II, III, and IV (30). (Genomovars II and IV have been renamed *Burkholderia multivorans* and *Burkholderia stabilis*, respectively [2, 62].) Within genomovar III, the highly transmissible strain (the ET12 strain [59]) expresses cable pili and its associated 22-kDa adhesin (46, 48). This is the strain most
B. cepacia infection in mice with CF

commonly cultured from CF patients in Canada and the United Kingdom and is the one most commonly associated with the cepacia syndrome (17, 32). Since this strain was shown to bind most strongly to mucins, to epithelial cells, and to tissue sections from lungs of CF patients (45, 47, 49), we have used this strain to develop an animal model of lung infection.

Given the difficulties in studying the pathogenesis of bacte-
rial infection in CF patients, murine models have been used as an experimental model system. CFTR-deficient (knockout) mice have been used to study experimental infection (7, 8, 10, 14, 19, 35). While these studies have provided important insights into the pathogenesis of CF lung disease, many have focused primarily on the acute responses to infection and some have relied on the use of agar-entrapped bacteria (14, 19) to assure the retention of bacteria in the lungs by mechanical means. In general, compared to wild-type mice, Cfr−/− mice demonstrate decreased bacterial clearance, an excessive inflammatory response, and significant mortality when challenged with pathogenic organisms including S. aureus, P. aeruginosa, and B. cepacia, indicating that the absence of CFTR may increase the susceptibility of mice to infection with these opportunistic pathogens. In contrast, several recent studies have found that Cfr−/− mice were able to clear P. aerugi-

nosa from their lungs as wild-type controls (7, 8, 35). These discrepancies may be explained by the complex genetic backgrounds of the mouse strains used, including the presence of alternative chloride channels, the different methods of bac-
terial delivery to the lungs, dietary factors, and the nutritional state of the mice. Thus, the relationship between CFTR ex-
pression, the pulmonary inflammatory response, and bacterial clearance remains uncertain.

Our goal was to develop a model of chronic pulmonary in-
fec tion with B. cepacia in mice with CF without the need for bacterial entrapment or use of immunosuppressive agents. We hypothesized that the ineffective inflammatory responses observed in CF patients would be manifest in this model and that the propensity for enhanced pulmonary inflammation and in-
jury would also depend on bacterial virulence. To investigate the latter, we used two strains of B. cepacia, one clinical isolate from the highly transmissible genomovar III (ET12) strain and an environmental type strain isolated from onion rot (ATCC 25416; genomovar I), to compare their virulence properties in mice. We compared the pulmonary inflammatory response to repeated administration of bacteria in both Cfr−/− and Cfr+/+ mice to judge the role of the Cfr−/− phenotype in enhancing susceptibility to lung infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A clinical isolate of B. cepacia BC7 was obtained from sputum of a CF patient at the Hospital for Sick Children in Toronto, Ontario, Canada. This patient died with the cepacia syndrome within 1 month of acquisition of the organism (45). Isolate BC7 is an ET12 strain, belong-
to genomovar III, has been classified as randomly amplified polymorphic DNA type 2, carries the epidemic DNA marker designated BCESM, and expresses surface cable pili (32, 46, 47). ATCC 25416 is an environmental type strain isolated from onion rot, belongs to genomovar I, and was purchased from the American Type Culture Collection (Manassas, Va.) (32). Both isolates were subcultured on brain heart infusion (BHI) agar (Becton Dickinson Co., Cockeysville, Md.) and single colonies were inoculated into 10 ml of tryptic soy broth (Difco Labs, Detroit, Mich.) and grown overnight on an orbital shaker at 150 rpm at 37°C. Bacteria were harvested by centrifugation at 6,000 × g for 10 min, and the bacterial pellet was suspended in sterile phosphate-buffered saline (PBS) to a concentration of 105 CFU/ml. Viable bacterial counts were measured by plating serial dilutions of bacteria on B. cepacia selective agar (20) or BHI agar plates.

Experimental animals. Long-surviving liquid-fed Cfr−/− mice (i.e., Cfr−/−) mice (24) and their littermate wild-type Cfr+/+ controls, age 6 to 8 weeks, were utilized in the study. Wild-type mice were also liquid fed during the experimental protocol to minimize differences that could occur due to diet or nutritional status. Genotyping was done as previously described (56), and only homozygotes (Cfr−/− and Cfr+/+) were used in this study. Mice were divided into three groups. In group 1, comprising 12 Cfr−/− and 11 Cfr+/+ mice, animals received only PBS. In group 2, comprising 12 Cfr−/− and 11 Cfr+/+ mice, animals were infected with isolate BC7. In group 3, comprising four Cfr+/+ and six Cfr−/− mice, animals were infected with isolate ATCC 25416. Experiments were carried out according to protocols approved by the animal care committee at the Hospital for Sick Children. The animals were housed in a clean conven-
tional area free of pathogens in sterile microisolator cages until they reached the required age. Littermate wild-type controls were maintained under identical conditions. Mice were transferred 24 h before the start of an experiment to a containment unit and housed in the same area throughout the experiment.

Infection of mice. Mice were anesthetized lightly using the inhalant anesthetic enflurane. To achieve intrapulmonary delivery, PBS (50 μl) or PBS containing B. cepacia (107 CFU) was instilled dropwise intranasally and allowed to be aspirated into the lungs. To ensure maximum delivery of bacteria into the lungs, mice were held with their mouths closed during the instillation. This technique was based on the results of preliminary experiments using 99mTc-labeled bovine serum albumin, which demonstrated maximum pulmonary delivery with minimal deliv-
ergy to the gastrointestinal tract (data not shown). Additional studies using nasal insillation of a suspension of iron-dextran followed by Pearl’s Prussian blue staining of the fixed and sectioned lungs demonstrated that the suspension was distributed equally to all lobes of the lungs and reached the alveoli (data not shown). Bacteria were administered on days 0, 3, 6, and 9, and the mice were sacrificed on day 18 by intraperitoneal injection of 0.3 ml of a 6.5-mg/ml solution of sodium pentobarbital and exsanguination.

BAL. Bronchoalveolar lavage (BAL) was conducted by instilling three 1-ml aliquots of sterile PBS via a cannula placed into the trachea and secured with ligatures. The average volume of BAL fluid recovered was 2.6 ml. The concentra-
tion of cells in BAL fluid was determined using a hemocytometer. Fifty microliters of BAL fluid was sedimented in a cytocentrifuge (Shandon Inc., Pittsburgh, Pa.), fixed with methanol, and stained using a modified Wright-Giemsa stain (Diff-Quick; Dade Diagnostics, Aquanda, Puerto Rico). The per-
centage of each cell type was determined by counting a total of 300 cells/slide. The cells in the remaining BAL fluid were sedimented by centrifugation and saved for analyses as outlined below. The supernatant was immediately mixed with a protease inhibitor cocktail (Boehringer Mannheim, Toronto, Ontario, Canada) and stored on ice until used. Lungs, blood, and spleens were also collected for analyses.

Flow-cytometric analysis. Cells obtained from the BAL fluid were incubated with 20% fetal bovine serum (FBS) for 30 min and washed with 10% FBS in PBS prior to addition of antibodies. As indicated in the legend to Fig. 6, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c and phycoerythrin-conjugated anti-major histocompatibility complex class II (MHC-II) (BD-Pharmingen Canada, Mississauga, Ontario, Canada) in 10% PBS in PBS, washed, and fixed with 1.6% paraformaldehyde. Two-color flow cytometry was performed using a FACScan flow cytometer equipped with CELLQuest software (Becton Dickinson, San Jose, Calif.). Macrophages were identified using a combi-
nation of light-scattering properties and surface expression of CD11c. The level of MHC-II surface expression was quantified on this population of cells. For assessment of oxidant production, cells were incubated with 10−5 M dihydro-
rhodamine (Molecular Probes, Eugene, Ore.) for 5 min at 37°C followed by fixation with 1.6% paraformaldehyde. The fluorescence of the reduction product, rhodamine 1-2-3, was evaluated by one-color flow cytometry as a measure of oxidant production as previously described (63). Surface CD4, CD8, and CD45 staining of the fixed and sectioned lungs demonstrated that the suspension was distributed equally to all lobes of the lungs and reached the alveoli (data not shown). Bacteria were administered on days 0, 3, 6, and 9, and the mice were sacrificed on day 18 by intraperitoneal injection of 0.3 ml of a 6.5-mg/ml solution of sodium pentobarbital and exsanguination.

Downloaded from http://iai.asm.org/ on November 9, 2017 by guest.
and/or B. cepacia selective agar plates. The number of CFU was determined after 72 h of incubation at 37°C. Identity of the bacteria recovered from animals was routinely confirmed by the Clinical Microbiology Department at the Hospital for Sick Children. When mice were infected with isolate BC7, a PCR with gene-specific primers for cfbA (48) was used to confirm the identity of the recovered bacteria.

Histopathological evaluation. Lungs were inflated with air, flushed via the pulmonary artery with PBS followed by 5% paraformaldehyde, and then fixed by immersion in 10% neutral buffered formalin overnight. Tissues were processed and embedded in paraffin, and 5-μm thick sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Giemsa, or trichrome stain. Slides were blinded for genotype of the mice and the treatment given and scored by a veterinary pathologist using a semi-quantitative scale in the range of 0 to 5 (10). Zero on this scale indicated no inflammatory change, while 5 represented severe inflammation with tissue destruction.

Immunofluorescence detection of B. cepacia in lungs and BAL cells. Lung sections from each mouse were deparaffinized and rehydrated in graded alcohol and water. Sections were heated in 10 mM sodium citrate buffer, pH 6.0, for 90 s at 121°C under pressure for antigen retrieval (52). Sections were washed with water, equilibrated in Tris-buffered saline (10 mM Tris buffer [pH 7.5] containing 0.15 M NaCl) and blocked with 5% normal donkey serum for 2 h at room temperature. Sections were incubated overnight at 4°C with polyclonal antibody R418 (1:1,000 dilution), which recognizes B. cepacia of all genomovars (44), and washed to remove excess antibody, and the bound antibody was detected by anti-rabbit immunoglobulin G conjugated with Cy3 fluorophore (1:250 dilution) (Jackson Immunolab Research Lab, West Grove, Pa.). Sections were counterstained with Mayer’s hematoxylin. When BAL fluid was used, cells were harvested by cytocentrifuge onto a slide, fixed in cold methanol, blocked with 5% normal donkey serum, and treated with anti-B. cepacia antibody R418 as described above.

Measurement of cytokines by ELISA. The levels of murine tumor necrosis factor alpha (TNF-α), KC/N51, gamma interferon (IFN-γ), and macrophage inflammatory protein 2 (MIP-2) in BAL fluid were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn.). All samples were analyzed in triplicate in a blinded fashion and compared with known standards.

EMSA. Nuclear levels of transcription factors NF-κB and CREB were measured by electrophoretic mobility shift assays (EMSA) essentially as described previously (38). In brief, 5 μg of protein from nuclear extracts of whole lungs was preincubated with nonspecific DNA competitor poly(dI-dC) (5 mg; Pharmacia, Piscataway, N.J.) for 10 min at room temperature. The 32P-radioabeled probe containing the NF-κB site of the murine TNF-α gene promoter (mTNF-α B3) was incubated for an additional 20 min at room temperature. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide (60:1 cross-link) Tris-glycine gel, and autoradiographs were prepared by exposure at −80°C using a Kodak X-OMAT film. To demonstrate specificity of the protein-DNA complex, a 125 M excess of unlabelled probe was added to the nuclear extract before adding the radiolabeled probe. The sequence of the plus strand of the oligonucleotide is 5′-AAACCAGGCGGTCCTTCCTC-3′.

Statistical analysis. Data that are normally distributed are expressed as mean values ± standard errors of the means (SEM). For these data, an unpaired Student t test with Bonferroni correction for multiple comparisons or analysis of variance (ANOVA) with correction for multiple comparisons (Sheffe) was used for statistical comparison of sample means as indicated in the figure legends. Nonparametric analysis using the Wilcoxon rank test was conducted on data that were not normally distributed. For these data, the medians and ranges of the values are illustrated. A P value of <0.05 was considered to be significant.

RESULTS

Initial deposition of B. cepacia in the lungs of Cfr−/− and Cfr+/+ mice. To establish that intranasal instillation efficiently delivered bacteria to the lungs of experimental mice, 50 μl of PBS containing 107 CFU of B. cepacia isolate BC7 was instilled once intranasally into three Cfr−/− and three Cfr+/+ mice. Mice were sacrificed 2 h later, and the lungs were harvested, homogenized, serially diluted, and plated on B. cepacia isolation agar to determine the number of viable bacteria. There was no observed difference between the three Cfr−/− and the three Cfr+/+ mice in the initial deposition rate, which was consistently over 10% (i.e., >106 CFU/lung). Bacteria were detected by the anti-B. cepacia antibody in all regions of the lungs (data not shown). Control mice that were given only intranasal PBS demonstrated no immunoreactivity with the anti-B. cepacia antibody.

Establishment of chronic pulmonary infection with B. cepacia. The next objective was to establish a more chronic pulmonary infection with B. cepacia in mice with a method that permitted physiologically relevant adhesive interactions between the bacteria and respiratory epithelium without the need for bacterial entrapment in agar beads. To accomplish this, sterile PBS or B. cepacia (107 bacteria per mouse) was administered on days 0, 3, 6, and 9 and the mice were sacrificed on day 18. Two strains of B. cepacia, a clinical isolate from the highly transmissible genomovar III, ET12 strain and an environmental type strain isolated from onion root (ATCC 25416; genomovar I), were used to compare their virulence properties in mice. Due to technical difficulties with the administration of the anesthetic, 1 to 3 mice died in each group during the intranasal instillation procedure, and these mice were removed from the study leaving 9 Cfr−/− and 10 Cfr+/+ mice in group 1 (PBS), 10 Cfr−/− and 16 Cfr+/+ mice in group 2 (B. cepacia isolate BC7), and 3 Cfr−/− and 4 Cfr+/+ mice in group 3 (B. cepacia isolate ATCC 25416). Two of the 16 Cfr−/− mice in group 2, which were infected with isolate BC7, died within a few hours after the fourth instillation on day 9, and at necropsy the lungs from both of these mice were characterized grossly by complete consolidation. The bacterial loads in these two mice were 2.3 × 108 and 9.7 × 107 CFU/g of lungs at the time of death. All of the remaining mice were sacrificed on day 18 and were used to generate data presented in this report.

The weights of the Cfr−/− mice (mean = 15.3 ± 0.9 g) were slightly less than those of Cfr+/+ controls (mean = 17.6 ± 0.8 g) at the beginning of the experiment. Both Cfr−/− and Cfr+/+ mice treated with PBS gained weight (mean weight gains for Cfr−/− and Cfr+/+ mice, 2.8 ± 0.2 and 3.1 ± 0.3 g, respectively). In contrast, Cfr−/− mice infected with isolate BC7 gained less weight during the experiment (1.6 ± 0.2 g) than Cfr+/+ controls (2.9 ± 0.4 g). Additionally, the Cfr−/− mice treated with the BC7 strain appeared lethargic compared to wild-type controls. Both the Cfr−/− and Cfr+/+ mice infected with isolate ATCC 25416 gained weight (mean weight gains for Cfr−/− and Cfr+/+ mice, 2.7 ± 0.3 and 3.0 ± 0.4 g, respectively) and appeared well.

Persistence of viable B. cepacia in the lungs. To determine the extent of bacterial persistence, lungs from each animal were dissected under aseptic conditions, homogenized, and plated on Burkholderia cepacia selective agar (BCSA) or blood agar plates. The lungs of all Cfr−/− animals that had received isolate BC7 harbored from 1 × 104 to 5 × 108 (median of 5.3 × 108) CFU of viable bacteria/g of lungs. In contrast, only three out of the eight Cfr+/+ mice infected with B. cepacia isolate BC7 had viable bacteria in their lungs and the counts were very low (<106 CFU/g). The other five Cfr+/+ mice had no detectable B. cepacia. By the Wilcoxon rank test, the difference between mouse groups was statistically significant (P < 0.001). None of the mice infected with isolate ATCC 25416 yielded positive lung cultures. Thus, even 9 days after the last exposure of mice to bacteria, ET12 strain BC7 was more persistent than environmental type strain ATCC 25416, and Cfr−/− mice were
more susceptible to infection than Cfr<sup>+/+</sup> mice. No viable <i>B. cepacia</i> organisms were found in the spleen, blood, or BAL fluid of either Cfr<sup>−/−</sup> or Cfr<sup>+/+</sup> mice infected with either BC7 or ATCC 25416.

**Instillation of <i>B. cepacia</i> results in bronchopneumonia.** On visual inspection, the lungs of both Cfr<sup>+/+</sup> and Cfr<sup>−/−</sup> mice treated with PBS alone or <i>B. cepacia</i> ATCC 25416 were grossly normal. Lungs of Cfr<sup>+/+</sup> mice infected with BC7 appeared healthy, with only occasional areas of atelectasis. In contrast, the lungs from Cfr<sup>−/−</sup> mice infected with BC7 were found to be friable and showed diffuse areas of consolidation and atelectasis. The lungs of both Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice infected with ATCC 25416 demonstrated minimal evidence of pathology.

By histological examination, Cfr<sup>+/+</sup> mice given PBS alone showed no evidence of inflammation of airway or lung parenchyma (Fig. 1a). Bronchi and bronchioles were lined by a constitutively normal population of ciliated and nonciliated (Clara) columnar epithelial cells with randomly distributed PAS-positive mucus-secreting cells (Fig. 1b). Mice of genotype Cfr<sup>−/−</sup> treated with PBS (not presented) also exhibited mostly normal lungs, although a few small, scattered areas of peribronchial and perivascular mononuclear cell cuffing were noted. Occasionally there were also small random multifocal patches of interstitial thickening characterized by fibroblast hypertrophy and mono- and polymorphonuclear inflammatory cell infiltration (data not shown). Thus lungs from Cfr<sup>−/−</sup> mice contained minor inflammatory changes in the absence of infection, presumably due to the effects of repeated instillation of PBS. Cfr<sup>+/+</sup> mice infected with isolate BC7 or ATCC 25416 were similar to each other in showing mostly normal and functional lungs, as seen in control, PBS-treated mice. However, in a few scattered areas there was peribronchial and perivascular inflammation (Fig. 1c). The epithelia of bronchioles were normal, but there were occasional small areas of hypertrophy of resident interstitial cells with associated infiltration by mononuclear inflammatory cells, consistent with pneumonitis (Fig. 1d). Therefore both BC7 and ATCC 25416 appeared to have caused a mild inflammatory response in Cfr<sup>−/−</sup> mice.

The histological appearance of the lungs of Cfr<sup>−/−</sup> mice treated with isolate BC7 was considerably different. There were almost no normal areas of lung remaining, and in many
regions there was complete pneumonic consolidation (Fig. 2a). Moderate-to-severe infiltration by lymphoplasmacytic cells was observed in most of the peribronchiolar and perivascular spaces. Epithelia of the affected airways exhibited striking Clara cell hyperplasia, prominent PAS-reactive domed apical hypersecretion, and mucus-like material over the luminal surface (Fig. 2b and c). The majority of the parenchyma was characterized by moderate-to-severe infiltration of alveolar

FIG. 2. Representative lung sections of Cftr<sup>−/−</sup> mice after repeated exposure to <i>B. cepacia</i> isolate BC7. (a, c, d) Hematoxylin- and eosin-stained sections; (b) PAS/Alcian blue-stained section; (e) section stained with Giemsa. The histological sections demonstrate hypertrophy of PAS-positive cells in bronchiolar epithelia, hypertrophy of Clara cells, mucus retention in airways, inflamed parenchyma characterized by marked hypertrophy of resident interstitial cells with infiltration by macrophages and neutrophils, and consolidation of alveolar airspaces by an exudation of inflammatory cells and debris.
septa by a mixed population of inflammatory cells composed predominantly of macrophages with some neutrophils (Fig. 2d). The airspace epithelium showed marked hypertrophy of type II pneumocytes and exudation by foamy vacuolated macrophages (Fig. 2e). None of the sections showed evidence of fibrosis. In contrast, Cfr<sup>−/−</sup> mice infected with isolate ATCC 25416 did not show any marked pathological changes and resembled Cfr<sup>+/+</sup> mice infected with the same isolate (not illustrated). Therefore clinical isolate BC7, but not isolate ATCC 25416, elicited a severe inflammatory response only in Cfr<sup>−/−</sup> mice. These observations may reflect differences between strains or the importance of bacterial virulence factors.

To quantitate these changes, the lung sections were examined by a veterinary pathologist who was blinded to the genotype of the mice and the treatment given. Severity scores for each mouse are shown in Table 1. ATCC 25416-infected Cfr<sup>+/+</sup> and Cfr<sup>−/−</sup> mice and BC7-infected Cfr<sup>−/−</sup> mice showed very-mild-to-moderate inflammation in the lungs, with the score ranging from 1 to 3. Cfr<sup>−/−</sup> mice infected with isolate BC7 showed moderate-to-severe bronchiolitis and pneumonia, with the score consistently averaging between 4 and 5. Thus the ET12 strain (isolate BC7), in addition to its greater pulmonary persistence 9 days after the last nasal instillation, also caused much more severe inflammation than did the environmental type strain (ATCC 25416).

**Localization of B. cepacia in the lungs.** To determine the anatomical site of the persistent bacteria, the lungs were examined by immunofluorescence microscopy using an antibody specific for B. cepacia. For both genotypes, mice that were infected with isolate ATCC 25416 harbored no bacteria in their lungs, despite evidence of mild inflammation. In contrast, BC7-infected Cfr<sup>−/−</sup> mice demonstrated bacteria in the consolidated and inflamed peribronchiolar and perivascular areas (Fig. 3a and b) and in the thickened alveolar septa and in areas of consolidation (Fig. 3e and f). Cfr<sup>−/−</sup> mice also showed bacteria in inflamed peribronchiolar areas (Fig. 3c and d) and thickened alveolar septa (Fig. 3g and h), but the density was much lower that for that for Cfr<sup>−/−</sup> mice.

**Characteristics of BAL fluid cell populations.** The cellular characteristics of BAL fluid were examined 9 days after the final instillation of bacteria. The total number of cells present in BAL fluid was significantly greater for Cfr<sup>−/−</sup> than for Cfr<sup>+/+</sup> mice treated with isolate BC7, and numbers for both mouse types were greater than those for the corresponding mice treated with PBS or isolate ATCC 25416 (Fig. 4a). In mice treated with PBS, macrophages comprised the majority of alveolar cells in both Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice (Fig. 4b). However, there was a significantly higher proportion of neutrophils (31.8%) in Cfr<sup>−/−</sup> than in Cfr<sup>+/+</sup> mice (14.1%; P < 0.02) infected with isolate BC7. The neutrophilia, as assessed by Wright-Giemsa staining of cytospins, corresponded to an increase in surface expression of myeloid differentiation antigen Ly-6G (GR-1) (13) in BAL cells as determined by flow cytometry (Fig. 4c).

Despite the increased percentage of neutrophils in BAL fluid of Cfr<sup>−/−</sup> mice, there was no significant difference in the degree of neutrophil activation between Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice infected with B. cepacia, as assessed by determining surface expression of β2 integrin CD11b/CD18 (Fig. 4d) or oxidative product (Fig. 4e). Importantly, these cells were capable of activation when removed from the milieu of the Cfr<sup>−/−</sup> lung, because exposure of the neutrophils recovered by lavage to phorbol-12-myristate-13-acetate, a potent neutrophil-activating agent, resulted in increased oxidant production (Fig. 4e). Thus, despite the presence of increased numbers of viable bacteria in the lungs and increased numbers of neutrophils in the BAL fluid of Cfr<sup>−/−</sup> mice, there was no evidence of enhanced neutrophil activation in the lung, which might be anticipated under these circumstances.

Analysis of the cells recovered in BAL fluid also revealed an increased proportion of lymphocytes from both Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice treated with BC7 (Fig. 4b). Further analysis of lymphocyte subtypes demonstrated that the majority (＞80%) of these cells were neither T nor B cells.

**Association of B. cepacia with the cells of BAL fluid.** In the cells recovered by BAL lavage from Cfr<sup>−/−</sup> mice infected with isolate BC7, a significant number of macrophages were associated with immunoreactive B. cepacia (Fig. 5a). In contrast, in comparable Cfr<sup>+/+</sup> mice, there were relatively fewer macrophages associated with bacteria (Fig. 5b), suggesting that alveolar macrophages from Cfr<sup>+/+</sup> mice may be more efficient in phagocytosing bacteria than those from Cfr<sup>−/−</sup> mice.

**Assessment of macrophage activation.** To assess the extent of macrophage activation, the levels of surface expression of F4/80 and MHC-II molecules (markers of macrophage activation) were determined by flow cytometry. CD11c expression was also measured as a marker for the total macrophage population. PBS control and BC7-infected Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice did not differ in their levels of CD11c surface expression (data not shown). A minor percentage (＜15%) of macrophages from all groups expressed F4/80, and no significant differences between Cfr<sup>−/−</sup> and Cfr<sup>+/+</sup> mice infected with isolate BC7 were noted (data not shown). In contrast, macrophages from BC7-infected Cfr<sup>−/−</sup> mice expressed 3.5 times more MHC-II than macrophages from similarly treated Cfr<sup>−/−</sup> mice (Fig. 6). These results indicate that, despite an enhanced pulmonary bacterial load, the alveolar macrophages
FIG. 3. Immunolocalization of *B. cepacia* in the lungs of *Cftr*<sup>1/1</sup> and *Cftr*<sup>2/2</sup> mice infected with isolate BC7. Paraffin sections (5 μm thick) were deparaffinized, rehydrated, heated in 10 mM sodium citrate buffer, pH 6.0, blocked with 5% normal donkey serum, and incubated overnight at 4°C with anti-*B. cepacia* antibody (diluted 1:1,000), and the bound antibody was detected by CY-3-conjugated anti-rabbit immunoglobulin G. (b and d) Bacteria in inflamed bronchoalveolar areas of *Cftr*<sup>2/2</sup> and *Cftr*<sup>1/1</sup> mouse lungs, respectively; (f and h) bacteria in infiltrated and inflamed parenchyma of *Cftr*<sup>2/2</sup> and *Cftr*<sup>1/1</sup> mouse lungs, respectively; (a, c, e, and g) hematoxylin- and eosin-stained sections corresponding to panels b, d, f, and h, respectively.
recovered from \(Cftr^{+/+}\) mice demonstrated suboptimal activation compared with those from \(Cftr^{-/-}\) mice.

**Transcription factor activation.** The transcription of many genes involved in acute inflammation (e.g., interleukin-1 [IL-1] and IL-6, TNF-\(\alpha\), and intercellular adhesion molecule 1 genes) is regulated by transcription factors such as CREB and NF-\(\kappa\)B (5, 27). To determine if the observed differences in the inflammatory response could be accounted for by variations in this aspect of inflammatory gene regulation, the nuclear translocation of these factors was determined in whole-lung extracts using EMSA. Low levels of nuclear translocation of CREB were present in all groups and did not differ between \(Cftr^{+/+}\) and \(Cftr^{-/-}\) mice (data not shown). The pattern of NF-\(\kappa\)B translocation was more complex (Fig. 7). In all \(Cftr^{-/-}\) mice treated with isolate BC7, increased levels of nuclear NF-\(\kappa\)B were present compared with those in PBS-treated controls. Notably, the levels of the CXC chemokine KC were elevated greater than twofold in \(Cftr^{-/-}\) mice compared to those in \(Cftr^{+/+}\) mice infected with BC7 (\(P = 0.02\) by \(t\) test). The elevation in the level of KC in \(Cftr^{-/-}\) mice is consistent with the neutrophil predominance in BAL fluid (Fig. 4b).

**DISCUSSION**

In the present study, we have demonstrated that a clinical isolate of \(B.\ cepacia\), BC7, belonging to the highly transmissible genovar III, ET12 strain (28, 59), from a case of CF persisted preferentially in the lungs of mice with CF 9 days after
the last instillation without the use of bacterium-immobilizing agents. The \( Cfr^{-/-} \) mice demonstrated an enhanced pulmonary inflammatory response but one that was apparently less effective than that for \( Cfr^{+/+} \) mice. On the other hand, an environmental type strain of \( B. cepacia \) (isolate ATCC 25416), which is predominantly a plant pathogen, did not persist or cause excessive inflammation in either \( Cfr^{-/-} \) or \( Cfr^{+/+} \) mice. Taken together, these observations suggest that the absence of functional CFTR in the lung creates a milieu that interferes with macrophage and neutrophil function and thereby impedes the clearance of a clinically relevant strain of \( B. cepacia \) from the lung. Additionally, the predilection for enhanced pulmonary inflammation and injury was dependent in part on bacterial virulence. It is noteworthy that only two \( Cfr^{-/-} \) mice died of lung infection, indicating that, similar to the human CF patient population, few mice with CF succumb to the initial stages of the infection. Rather, most mice with CF develop a more persistent infection that promotes an excessive and prolonged inflammatory response leading to progressive lung injury, as indicated by the pathological changes observed in infected \( Cfr^{-/-} \) mice.

There was a significantly increased proportion of neutrophils present within the airspace of \( Cfr^{-/-} \) mice infected with \( B. cepacia \) isolate BC7. The mechanism of this enhanced neutrophil infiltration and accumulation could involve enhanced recruitment, diminished clearance, or a combination thereof. Enhanced neutrophil recruitment could be due to the increased numbers of bacteria remaining in the lungs of mice with CF, which would promote the release of chemoattractant molecules such as the CXC chemokine KC from pulmonary macrophages, lymphocytes, and epithelial cells. However, it is also possible that intrinsic differences in the lungs of mice with CF predispose them to the increased recruitment and/or the decreased clearance of neutrophils. Several hypotheses have been proposed to account for the enhanced predilection of lungs of animals with CF for infection and excessive inflammation including (i) increased airway surface liquid absorption leading to depletion of the periciliary liquid layer and diminished mucociliary clearance (34), (ii) failure of airway epithelial cells in animals with CF to ingest bacteria and be sloughed, resulting in enhanced bacterial retention (40), (iii) abnormal surface properties of airway epithelial cells in animals with CF.
bacteria in the lungs of animals with CF, minimal evidence of enhanced activation, as assessed by oxidant production and surface expression of CD11b, was observed in the Cfr<sup>-/-</sup> mice. In this regard, it is noteworthy that in vitro exposure of neutrophils to lipopolysaccharide (LPS) from an ET12 strain of B. cepacia (isolate J2315, genomovar III) results in cell activation including NADPH oxidase activity and enhanced surface expression of CD11b (21). One possible explanation for these apparently discrepant observations is that live bacteria (as opposed to purified LPS) release toxins that prevent optimal neutrophil activation. Additionally, it is possible that factors present in the milieu of the lungs of animals with CF prevent complete activation of neutrophils. Because we studied these facets of neutrophil function at only a single time point (9 days) after the establishment of infection, a more comprehensive examination of neutrophil activation is now required.

Macrophages are also important effectors of lung defense as they are both bactericidal and have critical immune system-activating functions, including antigen presentation and orchestration of the pulmonary immune response by virtue of cytokine and chemokine production (65). We observed that, despite a much larger bacterial burden in the lungs of Cfr<sup>-/-</sup> mice, the levels of MHC-II surface expression, an indicator of macrophage activation, were much lower in these mice than in their wild-type counterparts. There are at least two possible explanations for these observations. One is that specific (but as yet uncharacterized) virulence factors of B. cepacia directly limit MHC expression and other aspects of macrophage activation as a means of evading the innate immune system. Similar evasion of host defenses has been demonstrated for other respiratory pathogens (36). A second possibility is that the unique milieu of the lungs of animals with CF may impede macrophage activation and microbicidal activity.

The localization of B. cepacia in the lungs of Cfr<sup>-/-</sup> mice to the alveolar septum in our studies is noteworthy and is in contrast to the usual situation in human CF patients, where chronic airway infection predominates (11). However, the localization of B. cepacia to the alveolar septum in human CF patients has recently been reported (44) and may reflect evasion by the virulent bacteria of host defense systems, allowing movement of the bacteria across the epithelial barrier and into the interstitial space. This translocation may presage the development of pneumonia and eventually systemic dissemina-

TABLE 2. Cytokine levels in BAL fluid

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mean cytokine concn (pg/ml of BAL fluid) for mice of indicated genotype ± SEM after treatment with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS BC7</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.3 ± 2.3 4.2 ± 1.4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>30.5 ± 9.6 20.7 ± 30.1</td>
</tr>
<tr>
<td>KC</td>
<td>203 ± 100 190 ± 118</td>
</tr>
<tr>
<td>MIP-2</td>
<td>80 ± 40 90 ± 24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistical analysis was by unpaired t test, * P = 0.02 for Cfr<sup>-/-</sup> mice versus Cfr<sup>+/+</sup> mice treated with BC7 and for Cfr<sup>-/-</sup> mice treated with BC7 versus Cfr<sup>-/-</sup> mice treated with PBS; ** P = 0.02 for Cfr<sup>-/-</sup> mice versus Cfr<sup>-/-</sup> mice treated with PBS; +++, P = 0.005 for Cfr<sup>-/-</sup> mice versus Cfr<sup>-/-</sup> mice treated with BC7.

<sup>b</sup> +/+, Cfr<sup>+/+</sup>; -/-, Cfr<sup>-/-</sup>; n = four to six mice for each group.
tion with the development of cepacia syndrome, a devastating condition associated with a high mortality (12, 60).

Animal models for CF have proven to be useful in studying the physiological significance of mutations in the Cfr locus that predispose animals to chronic lung infections and subsequent inflammatory lung injury. To date, various models have shown a predilection of the lungs of mice with CF for infection with several opportunistic pathogens including P. aeruginosa, Hae-
morphilus influenzae, S. aureus, and B. cepacia (10, 14, 19). However, to establish bacterial infection in rodent lungs, some investigators have used immobilizing agents such as agar beads (14, 19) which bypass colonization mechanisms such as interactions between bacterial adhesins and respiratory epithelial cells (42, 46, 47, 49, 50). Most studies to date have focused on the host response to infection with P. aeruginosa and S. aureus, while information regarding the host response to B. cepacia is more limited, in part because of the difficulties of establishing a pulmonary infection with this organism. One study, however, compared the acute responses to challenge with aerosolized B. cepacia or S. aureus, without the use of agar beads, in Cfr<sup>−/−</sup> and Cfr<sup>+/−</sup> mice (10). These Cfr<sup>−/−</sup> mice, generated by tar-
geted insertional mutagenesis of exon 10 (Cfr<sup>m1UNC</sup>), were repeatedly exposed to aerosolized S. aureus and B. cepacia in separate experiments. It is notable that the B. cepacia used in this study (J2315; genomovar III) was also an ET12 strain. Bacterial clearance was significantly impaired, and the his-
topathological abnormalities were more pronounced for both organisms in CFTR-deficient mice than in wild-type controls. Mice infected with S. aureus demonstrated bronchiitis and bronchiolitis, whereas those infected with B. cepacia demon-
strated severe bronchopneumonia. The pathological changes seen with B. cepacia are similar but more severe than those seen in our model. To achieve these results, the experimental protocol involved aerosolization of bacteria daily for an entire month. This represents a much greater bacterial load than that used in our study.

In contrast, when another strain of CFTR-deficient mice (Cfr<sup>m1UNC</sup>, S489X null mutant) was challenged with S. aureus, no differences in bacterial clearance between CFTR-deficient mice and the controls were demonstrated (55). In this study, no mechanical immobilizing agents were used to increase reten-
tion of the bacteria in the lungs. However, when these same Cfr<sup>m1UNC</sup> mice were given P. aeruginosa enmeshed in agar beads (19), an increased mortality and elevated levels of inflam-
matory cytokines (TNF-α, MIP-2, and KC) were noted early in the course of infection in mice with CF compared to controls. No differences in either bacterial burden or in the composition of inflammatory cells in BAL were noted, how-
ever, between mice with CF and wild-type control mice. In our study, pathological changes were much more severe in the Cfr<sup>−/−</sup> mice than in the Cfr<sup>+/−</sup> mice treated with B. cepacia, a difference that may reflect the repetitive-exposure regimen using intranasal instillation.

In our model, no viable B. cepacia organisms were found in the spleens or blood of either Cfr<sup>−/−</sup> or Cfr<sup>+/−</sup> mice infected with either B. cepacia clinical isolate BC7 (ET12 strain) or the environmental type strain (isolate ATCC 25416). This lack of systemic dissemination is in contrast to the report by Speert and colleagues, who infected IFN-γ knockout mice with B. multivorans (genomovar II) isolated from a patient with chronic granulomatous disease and recovered viable bacteria from the spleen (58). This discrepancy may reflect variations in the route of administration of the bacteria (intranasal versus intraperitoneal), differences in the pathogenicities of the two strains of B. cepacia (ET12 strain of genomovar III versus B. multivorans) used, or the generalized compromise of the immune system in the IFN-γ knockout mice compared to the apparently localized compromise of pulmonary host defenses in the CF knockout mice.

It is not clear why CF predisposes patients to acquisition of B. cepacia. Host lung factors undoubtedly play an important role, and B. cepacia infections usually occur in patients with lung damage due to chronic infection by P. aeruginosa (16, 30). Indeed, human CF patients are frequently infected by several different bacteria, and this polymicrobial infection may alter the host response to B. cepacia. Epidemic-like spread of B. cepacia usually occurs in a pattern reflecting close person-to-person social contacts, suggesting that recurrent exposure to the pathogen is important in increasing susceptibility to infection (15, 28, 37, 53). Our murine system with repetitive exposure to B. cepacia was designed to model this environment. It should be noted that even apparently immunocompetent patients can develop B. cepacia pneumonia if host defenses are overwhelmed (1, 29, 64).

Specific bacterial factors are also assumed to play an impor-
tant role in determining the clinical severity of B. cepacia infections in CF patients. Bacterial virulence factors that have been proposed include surface cable pili, which are expressed by some B. cepacia isolates of the ET12 strain and which mediate adherence to mucins and epithelial cells (46, 47, 49, 50), extracellular proteases (57), lipases (31), siderophores (9), hemolysins (22), LPS (21, 66), and melanin-like pigment (67). The ability of some strains of B. cepacia to enter epithelial cells and macrophages and to replicate intracellularly (3, 33, 43) and their resistance to phagocytic killing (67) are also likely to contribute to virulence.

Infection with B. cepacia remains an important clinical prob-
lem for CF patients in many centers, yet this pathogen and the host response to this infection remain poorly understood. In this study, we have reported the development and character-
zation of a murine model of chronic pneumonia due to B. cepacia that leads to bacterial persistence and chronic pulmo-
mary inflammation consistent with clinical CF disease. Impor-
tantly, we provide evidence that there is suboptimal activation of pulmonary neutrophils and macrophages in the milieu of the lungs of animals with CF that may contribute to bacterial persistence. Further studies using this model will allow a better understanding of the host-microbe interaction for B. cepacia and the ineffective, yet exaggerated, inflammatory response that characterizes this disease. Given the lack of adequate therapy for treating B. cepacia infection, this model may also allow for the development and testing of novel therapies to limit bacterial and immune-system-mediated lung damage.

ACKNOWLEDGMENTS

This work was supported by operating grants from the National Institutes of Health (P50 DK49096–06 SCORE) to G. P. Downey and


Editor: E. I. Tuomanen