Excessive Inflammatory Response of Cystic Fibrosis Mice to Bronchopulmonary Infection with Pseudomonas aeruginosa

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Abstract

In cystic fibrosis (CF), defective function of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells and submucosal glands results in chronic pulmonary infection with Pseudomonas aeruginosa. The pulmonary infection incites an intense host inflammatory response, causing progressive suppurative pulmonary disease. Mouse models of CF, however, fail to develop pulmonary disease spontaneously. We examined the effects of bronchopulmonary infection on mice homozygous for the S489X mutation of the CFTR gene using an animal model of chronic Pseudomonas endobronchial infection. Slurries of sterile agarose beads or beads containing a clinical isolate of mucoid P. aeruginosa were instilled in the right lung of normal or CF mice. The mortality of CF mice inoculated with Pseudomonas-laden beads was significantly higher than that of normal animals: 82% of infected CF mice, but only 23% of normal mice, died within 10 d of infection (P = 0.023). The concentration of inflammatory mediators, including TNF-α, murine macrophage inflammatory protein-2, and KCN/N5I, in bronchoalveolar lavage fluid in CF mice 3 d after infection and before any mortality, was markedly elevated compared with normal mice. This inflammatory response also correlated with weight loss observed in both CF and normal littermates after inoculation. Thus, this model may permit examination of the relationship of bacterial infections, inflammation, and the cellular and genetic defects in CF. (J. Clin Invest. 1997. 100: 2810–2815.) Key words: Pseudomonas aeruginosa • lung • cystic fibrosis • inflammation

Introduction

In cystic fibrosis (CF),1 defective function of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells and submucosal glands results in chronic disease of the respiratory tract manifested early in life by airway obstruction and recurrent infections of the lung and paranasal sinuses. The CF lung is particularly susceptible to Pseudomonas aeruginosa, and this organism plays a critical role in the development and progression of pulmonary disease in CF. Individuals with CF acquire an endobronchial infection that elicits an intense inflammatory response. This response is characterized by a marked influx of neutrophils into the lung, and elevations in inflammatory mediators such as TNF-α, IL-1β, IL-6, leukotriene B4 (LTB4), and IL-8 (1). Neutrophils are the predominant inflammatory cell in the lungs of patients with CF, even in individuals with mild pulmonary involvement (2). The inflammatory response in patients with CF appears to be excessive, and current management of pulmonary disease in CF is directed at the consequences of infection and inflammation (3). Moreover, some infants with CF appear to have an inflammatory response in the airways, even if infection is not detected (4).

Several hypotheses have been proposed to explain CF lung predilection for excessive inflammation and infection with P. aeruginosa. Some investigators suggest that the elevated salt content in the surface fluid of the CF airway renders human β-defensin-1 nonfunctional, eliminating the bactericidal activity of the respiratory epithelium (5, 6). A second hypothesis suggests that failure of the respiratory epithelial cells in the CF lung to ingest bacteria and be sloughed allows for P. aeruginosa retention at the endobronchial surface (7). A third possibility is that Pseudomonas adheres to epithelial cells in the CF airway in greater numbers because of the abnormal surface properties of the cells, thus leading to infection (8–10). Although details of the mechanism differ, all of these hypotheses predict that the basic defect in CF permits retention of bacteria at an otherwise sterile site, providing the stimulus for inflammation. If abnormal retention of bacteria by itself accounts for disease in the CF lung, then assuring retention of bacteria in the lung by mechanical means should eliminate the differences between the normal and CF airway. To test this hypothesis, we studied the responses of mice homozygous for the null S489X mutation and their normal littermates to instillation of agarose beads containing Pseudomonas to the airway. The histopathologic features of this animal model of chronic Pseudomonas endobronchial infection and inflammation closely mimic those present in the lungs of patients with cystic fibrosis (11). If abnormal bacterial retention accounts for excessive inflammation in CF, CF mice and their normal littermates should respond similarly to this challenge. In fact, the CF mice had greater mortality. This result suggests that the CF genotype is associated with excessive inflammatory response compared

1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; EIA, enzyme immunoassay; LTB4, leukotriene B4; mip-2, macrophage inflammatory protein-2; mTNF-α, murine TNF-a.
with the normal response, even if the initiating stimulus is similar.

Methods

Animals. Mice homozygous for the S489X (null) mutation of the CFTR and their normal littermates (wt/wt and S489X/wt) were studied (12, 13). The genotype of individual animals was established by polymerase chain reaction amplification of genomic DNA isolated from the animal’s tail as previously described (Perkins Elmer Corp., Norwalk, CT; 14). Pups were weaned and fed a liquid elemental diet (Peptamen®; Clinitest Nutrition Co., Deerfield, IL) ad libitum beginning at 10 d of life to avoid the intestinal obstruction characteristic in mice with this mutation (14). Sterile water was available at all times for control and experimental animals. All mice were maintained in microisolator units with inedible bedding, which has been shown to reduce the frequency of intestinal obstruction (13). With these treatments, most CF mice reach adulthood and have grossly normal lungs. Fully grown, the CF mice weighed 23.7±0.7 g, compared with 27.2±0.4 g for their normal littermates. Animals were studied when they were >90 d of age and >15 g body weight.

Experimental model of chronic P. aeruginosa infection. The agarose bead method of chronic Pseudomonas endobronchial infection developed by Cash et al. (11) and modified for mice by Starke et al. (15) was used to create chronic pulmonary infection in mice with several variations. In brief, agarose was mixed with tryptic soy broth (TSB) or TSB containing mucoid P. aeruginosa strain M57-15, a clinical isolate from a CF patient, and grown to late log phase. The agarose–broth mixture was added to mineral oil that was equilibrated at 50°C, rapidly stirred for 6 min at room temperature, and then cooled over 10 min. The agarose beads were washed once with 0.5% deoxycholic acid, sodium salt (SDC) in PBS, pH 7.4, once with 0.25% SDC in PBS, and four times with PBS. The beads were measured by light microscopy, and their diameter ranged from 77 to 126 μm. Quantitative bacteriology was performed on an aliquot of homogenized bead slurry to determine the number of CFU per ml (1.3×10^7 CFU/ml slurry). Sterile bead preparations were confirmed to be sterile.

Mice were anesthetized with 2.5% Avertin (2% 2,2,2-tribromoethanol 2% tert-amyl alcohol 0.9% NaCl), placed in dorsal recumbency, and the ventral central area was surgically prepared. A transverse skin incision was made, and the trachea was visualized by blunt dissection. Transtracheal insertion of a 22G 1-in catheter was used to instill 50 μl of a 1:10 dilution of the bead slurry into the right lung (6.1×10^7 CFU/mouse). The animal research protocol was reviewed and approved by the Case Western Reserve University Institutional Animal Care Committee.

Experimental protocol. In initial experiments, normal and CF mice underwent intratracheal injection with sterile agarose beads or beads embedded with Pseudomonas; cohorts were monitored for 10 d after inoculation. Animals were weighed 3 d after inoculation with either sterile or Pseudomonas-laden beads and daily thereafter.

In subsequent experiments, 18 CF mice and 22 normal littermate controls were inoculated with Pseudomonas-laden beads and weighed 3 d afterward. The mice were then killed by carbon dioxide narcosis and exsanguinated by cardiac puncture. The lungs were perfused with 1.0 ml PBS via the right ventricle. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea in situ with a 22G 1 1/2-in bead-tipped feeding needle, instilling 1.0 ml of sterile PBS three times, and collecting the fluid by gentle aspiration. The total lavage fluid recovered from all animals was >2.5 ml. The fluid was centrifuged for 10 min at 4°C, and the supernatants were stored at −20°C, some aliquots with 100 μl PMSF and 5 mM EDTA. The pellet was suspended in 1.0 ml PBS, and a cell count and leukocyte differential was performed. Cell counts, quantitative bacteriology, and inflammatory mediators were measured in BAL fluid by investigators without knowledge of the origin of the samples. A normal control and two CF animals died of postoperative complications; a CF mouse and a normal littermate failed to receive the full bacterial inoculum because of technical problems. These animals were not included in the analysis.

Lung histopathology. After BAL, the lungs were inflation-fixed in 2% paraformaldehyde in PBS for 48 h, and then embedded in paraffin. The lung was cut once midsagittally and embedded in paraffin. Tissue sections were cut throughout the entire tissue block such that 5-μm sections were taken at regular intervals that encompassed the full craniocaudal range of sections. Sections were stained with hematoxylin and eosin using standard techniques, and were examined for histopathologic changes.

Quantitative bacteriology. The lungs and bronchi of 12 normal and 10 CF mice inoculated with agarose beads embedded with P. aeruginosa 3 d previously were excised aseptically, homogenized in 40 ml normal saline (pH 7.4), and samples of the lung and BAL homogenates were cultured quantitatively by serial dilution on tryptic soy agar plates (16). The upper limit of quantitation was 1.0×10^7 CFU/ml, which was reached or exceeded by two CF mice.

Spleens were removed and homogenized in 1.0 ml sterile PBS. A 10-μl aliquot of the homogenate was applied to tryptic soy agar plates. Blood was also collected from all animals, and streaked on tryptic soy agar plates. The plates were incubated at 37°C, and were inspected for P. aeruginosa colonies after 24 h.

Analysis for proinflammatory cytokines in bronchoalveolar lavage fluid. Protease inhibitors were added to the BAL samples immediately after collection, and murine tumor necrosis factor-α (mTNF-α), interleukin-1β (mIL-1β), interleukin-6 (mIL-6), murine macrophage inflammatory protein-2 (mIP-2), and mouse KC/NS1 (KC) were measured using commercially available sandwich enzyme immunoassays (EIA) according to the manufacturers’ recommended protocols (R&D Systems, Minneapolis, MN). The limits of detection for the cytokines were 5.0 pg/ml. The BAL supernatants were assayed in duplicate, and the values were corrected for the respiratory epithelial lining fluid (ELF) volume recovered by measuring urea dilution (17).

Leukotriene-B4 assay of bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid was analyzed for the presence of LTB4 using a competitive EIA as previously described (Cayman Chemical Co., Ann Arbor, MI; 18). Aliquots (100 μl) of BAL supernatants were evaporated by vacuum centrifugation and suspended in 100 μl ice-cold methanol:water (1:1). To these samples, 200 μl of the EIA buffer was added and assayed by standard techniques (18). The values of LTB4 were assayed in duplicate, compared with known standards, and corrected for the volume of respiratory ELF recovered.

Statistical analysis. Data are expressed as the mean±SEM. Data comparing the clinical status of CF mice and normal littermates were analyzed by an unpaired, two-tailed Student’s t test, and the relationships between the infected animal’s weight loss and the inflammatory response in the lung were assessed by Pearson correlation coefficient. Fisher exact test was used to compare the survival of normal and CF mice after inoculation with either sterile or Pseudomonas-laden beads. Concentrations of inflammatory cells, proinflammatory mediators, and bacterial burden in the lungs of normal and CF mice were compared using a nonparametric ANOVA using the Kruskall-Wallis test (19).

Results

Growth. Fig. 1 shows the initial body weights of the CF mice and normal littermates at the onset of the experiments, and the percent change in body weight in these mice 3 d after inoculation with either sterile or Pseudomonas-laden agarose beads. Although the range of weights is identical for both groups, the CF mice were smaller than the normal animals. The CF mice (n = 25) treated with the Pseudomonas-laden beads lost significantly more weight 3 d after inoculation than their normal littermates (n = 51; P = 0.0004). The cumulative weight loss of both CF (n = 5) and normal mice (n = 11) after inoculation with sterile beads was similar.

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Survival. Fig. 2 illustrates the cumulative survival of mice that received sterile or *Pseudomonas* -laden agarose beads. The mortality of CF mice inoculated with *Pseudomonas*-laden beads was significantly higher than that of normal animals (*P* = 0.023); 82% (9/11) of the infected CF mice died by 5 d after infection, whereas only 23% (6/26) of normal mice died within 10 d of infection. All mice that received sterile beads survived 10 d after inoculation.

Pathology. The gross lung pathology of CF mice and their normal littermates 3 d after inoculation with *P. aeruginosa* -laden agarose beads ranged from small punctate off-white nodules throughout the lung to frank consolidation of lung tissue. Histopathology sections of these mice were observed to have areas of leukocytic infiltration involving the airways, bronchus-associated lymphoid tissue, and adjacent parenchyma. Early fibrosis was evident in some of the more severely affected areas of lung (Fig. 3). Beads were seen in some airways, surrounded by neutrophils (Fig. 3 c). The right lung was preferentially inoculated with the beads, but in some animals there was some pathology evident in the left lungs due to spillover from the right lung. Those mice that survived 10 d after *Pseudomonas* inoculation were observed to have milder pulmonary inflammation than those mice killed 3 d later. Sterile agarose beads were associated with minimal inflammation 3 d after inoculation into mice (data not shown).

Bronchoalveolar lavage. Bronchoalveolar lavage fluid from CF patients contains numerous neutrophils and products from the inflammatory cells. We examined cell composition in the BAL samples of normal and CF mice infected with *P. aeruginosa*. The number of inflammatory cells in the lung was markedly elevated in mice inoculated with *Pseudomonas*-laden
beads. This inflammatory cell response correlated directly \( (r = -0.43, P < 0.02) \) with the weight loss of both CF and normal mice after inoculation with \textit{Pseudomonas}-laden beads (Fig. 4). In separate experiments, there were no significant differences in cytokine and cell concentrations from BAL fluid in untreated animals and mice inoculated with sterile agarose beads (data not shown).

Infection with \textit{P. aeruginosa} stimulates production of other inflammatory mediators, including LTB\(_4\) released predominantly by phagocytes, mTNF-\(\alpha\), and mIL-1\(\beta\) (predominantly by mononuclear cells), and mIL-6 (predominantly by epithelial cells). Normalized levels of these mediators were measured in BAL samples of normal and CF mice 3 d after treatment with sterile or \textit{Pseudomonas}-laden beads. All of the inflammatory mediators measured were elevated in the BAL fluid from infected CF mice compared with their infected normal littermates (Table I). In particular, the concentrations of mTNF-\(\alpha\) \( (P = 0.049) \), mip-2 \( (P = 0.0078) \), and KC \( (P = 0.001) \) in the BAL fluid from CF mice were significantly higher than those measured in normal littermates after inoculation with \textit{Pseudomonas}-laden beads. Murine IL-1\(\beta\) levels were higher in the infected CF mice, but the levels detected did not achieve statistical significance compared with infected normal mice \( (P = 0.0535) \). In turn, normal mice inoculated with \textit{Pseudomonas}-laden beads had higher concentrations of these mediators compared with mice that were not infected or received sterile beads. By day 3 after sterile bead inoculation, only background levels of IL-1\(\beta\) and low levels of mip-2 could be measured at all under our assay conditions, and these levels were similar for CF mice and their littermate controls (data not shown).

**Bacteriology.** Lung homogenates yielded \textit{P. aeruginosa} in pure culture, and Fig. 5 shows the results of quantitative bacteriology of the lungs from CF \( (n = 10) \) and normal \( (n = 12) \) mice.

<table>
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<tr>
<th>Table I. Inflammatory Indices in the Lungs of CF and Normal Mice 3 d After Intratracheal Inoculation with Beads Laden with \textit{P. aeruginosa}</th>
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<td>Leukocytes (10(^6) cells/ml)</td>
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<td>2.5±0.4</td>
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<td>Neutrophils (10(^6) cells/ml)</td>
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<td>1.7±0.5</td>
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<td>LTB(_4) (ng/ml)</td>
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<td>TNF-(\alpha) (ng/ml)</td>
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<td>13.7±3.3*</td>
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<td>IL-6 (ng/ml)</td>
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<td>mip-2 (ng/ml)</td>
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<td>KC (ng/ml)</td>
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<td>11.1±2.2*</td>
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\( P < 0.05 \) based on nonparametric Kruskal-Wallis ANOVA.

Figure 4. The relationship between the inflammatory response and clinical status of CF and normal mice. Inflammatory cells were collected by BAL from CF mice and normal littermates 3 d after inoculation with \textit{P. aeruginosa}. Solid symbols, CF mice; open symbols, normal mice. Correlation coefficient \( (r) \) of \(-0.43 \) \( (P < 0.02) \).

Figure 5. Bacterial burden in the lungs of CF and normal mice after inoculation with \textit{P. aeruginosa}. Animals underwent intratracheal inoculation with \textit{Pseudomonas}-laden beads \( (6.8 \times 10^4 \text{ cfu/mouse}) \), and 3 d after treatment, bacteria was isolated from both lungs and BAL homogenates. The number of CFUs was determined by serial dilution on tryptic soy agar plates. Bacterial load in the lungs of infected normal and CF mice were compared using the Kruskall-Wallis test, and did not differ between groups.
mice. Bacterial colony counts of \textit{P. aeruginosa} from the lungs were similar in the CF mice and their normal littermates (3.4±1.6 × 10^6 and 3.1±1.6 × 10^6 CFU/mouse, respectively). There was no growth from the blood of any mouse after inoculation with the \textit{Pseudomonas}-laden beads, and only three animals—one CF mouse and two normal mice—had splenic homogenate positive for \textit{P. aeruginosa} 3 d after inoculation.

**Discussion**

Knockout mice homozygous for the S489X mutation of the CFTR have markedly increased mortality when challenged with an intratracheal instillation of agarose beads laden with \textit{P. aeruginosa}. The increased mortality in CF mice is associated with an increased pulmonary inflammatory response, but also with a comparable lung burden of bacteria when measured 3 days after infection (before the CF mice begin to die). Premature death of CF mice appears to be due to bronchopneumonia and not to sepsis, as no blood cultures and only one splenic homogenate yielded \textit{P. aeruginosa}. Thus, it appears that the lung inflammatory response to \textit{Pseudomonas} is excessive.

Animal models of CF do not spontaneously develop pulmonary disease. Even as CF mice are rescued from intestinal obstruction and live longer, they do not appear to be predisposed to pulmonary infection. Mice with a null mutation of the gene encoding CFTR that produce some normal protein (Edinburgh mice) have a greater probability than non-CF mice of developing infection after repeated aerosol challenges of \textit{Staphylococcus aureus} or \textit{Burkholderia cepacia} (20). The response of these mice to \textit{P. aeruginosa}, the most common and distinctive pathogen in the CF lung, was not reported.

In the airways of CF patients, the basal potential difference across epithelia is increased, with an increased amiloride-sensitive component. Agents that elevate intracellular cyclic AMP, like forskolin or isoproterenol, fail to elicit the change in trans-epithelial potential difference that occurs in normal subjects (21). Tracheas removed from mice homozygous for the S489X mutation, however, have transepithelial potential difference measurements across the airway comparable to those of normal mice, including a normal chloride secretory response to treatment of the tracheal epithelial surface cyclic AMP sensitiveos. This chloride secretion occurs via a calcium-regulated channel (22), for it can be inhibited by an intracellular calcium chelating agent like 1,2-bis(o-Aminophenoxo)ethane-N,N,N’-tetracetic acid. It has been suggested that CF mice compensate for the lack of CFTR in the airways with an alternative chloride channel, and although it is uncertain whether the salt content of the epithelial lining fluid is increased in the lungs of CF mice, the relatively normal airway electrophysiology suggests that the ionic composition of the epithelial lining fluid may also be close to normal (23). Our results, however, indicate that the CF defect in these mice, insufficient to produce detectable abnormalities in airway electrophysiology or spontaneous pulmonary infections, is sufficient to produce increased morbidity and mortality when the mice are challenged with \textit{Pseudomonas}. In this model, the bacteria are retained in the lung mechanically, and the model does not depend on impaired clearance of instilled bacteria by the host to establish infection. Instead, the agarose beads permit establishment of a chronic infection in both normal and CF mice, and produce comparable bacterial colony counts at 3 d in both. Thus, the increased mortality of the CF mice suggests that these animals have an exaggerated inflammatory response to \textit{P. aeruginosa}.

The lungs of mice after intratracheal inoculation with the \textit{Pseudomonas}-laden beads resembles the pulmonary disease characteristic of CF in many important ways. Histopathological examination of the lung shows an intense peribronchial and endobronchial inflammatory response with a lesser component of alveolar involvement, which is primarily mediated by neutrophils. Neutrophils are the prominent inflammatory cell in the lungs of patients with CF, even in individuals with minimal pulmonary involvement (2). Animals so infected make antibodies against \textit{P. aeruginosa} that are initially opsonic, but with time, the antibody response becomes less effective and may even be blocking. The response of animals in this model to anti-\textit{Pseudomonas} vaccines and to antiinflammatory therapy is similar to that observed in patients with CF (16, 24, 25). No animal model for the lung disease of CF is perfect, however, and the short time course over which infection and inflammation develop in the animals and the eventual decline in bacterial counts in normal animals, requiring repeated inoculations to sustain infection over a period of months, are important differences. Nevertheless, this model could illuminate the host response to chronic infection of the airways with \textit{Pseudomonas}. In this model, CF mice had an increased inflammatory response 3 d after inoculation, but also had comparable bacterial colony counts. The pulmonary infiltrate in both normal and CF mice is predominantly neutrophilic. The remarkably high levels of proinflammatory mediators, especially chemoattractant cytokines (present in the lungs of CF mice in this model), is similar to that observed in infants and young children with CF, even when controlled for bacterial burden (26). Proinflammatory cytokines and neutrophil markers are markedly increased in the lungs of CF mice (as in CF patients), and may perpetuate the robust inflammatory response (4, 27, 28). Excessive pulmonary infection and inflammation have systemic effects, manifest by weight loss, and ultimately cause excess deaths of CF mice compared with the control animals.

In this model, \textit{P. aeruginosa} is held in the lung by mechanical means, thus bypassing initial \textit{Pseudomonas} attachment and retention. Consequently, this model does not test the ability of the animals to clear bacteria from the lung or to establish the relative importance of antibacterial factors or ingestion of bacteria in early development of pulmonary disease in CF. Rather, it permits us to examine the host response to \textit{Pseudomonas}, which appears to be excessive in CF mice, and may be a critical determinant for pulmonary disease progression. These data indicate that increased inflammatory response contributes to pulmonary disease genesis, and suggest that pulmonary disease in CF may result from factors independent of the organism’s impaired clearance. If the normal electrophysiology of the trachea in CF mice and their normal littermates predicts a normal salt concentration in the epithelial lining fluid, then excessive inflammation may arise independent of airway surface fluid salt content and other factors that may contribute to pulmonary disease in CF.

Although inflammation has been perceived as a response to an infectious stimulus, studies in young children with CF diagnosed by neonatal screening suggest some dysregulation of the inflammatory response that may be directly related to the molecular defect (4). Investigators have reported high levels of proinflammatory cytokines and neutrophils in the bronchoalveolar lavage fluid of infants with CF, and several children had
pulmonary inflammation even in the absence of evidence of infection (4). Thus, it is possible that inflammation may occur independent of infection, or that a relatively minor infection may induce a robust and persistent inflammatory reaction in the CF lung. Recent histopathological studies of CF mice raised in pathogen-free conditions revealed a greater number of lymphocytes in the airway submucosa compared with wild-type littermates (29). Our CF animals inoculated with sterile beads, however, did not have an excessive inflammatory or death rate.

The results of these and earlier studies suggest that at least two factors lead to the propensity of patients with CF to develop chronic endobronchial infection and inflammation: (a) retention of bacteria in the lung, and (b) an intense, excessive inflammatory response to the organism. Thus, in order to understand the pathogenesis of pulmonary disease characteristic of CF, we need to examine not only the impaired clearance of the bacteria, but also the excessive host response to P. aeruginosa.

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