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Microbiology of Airway Disease in Patients with Cystic Fibrosis†

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INTRODUCTION

The syndrome of cystic fibrosis (CF) of the pancreas was first described in 1938 by Anderson (3). She described this syndrome in children who failed to thrive despite frequent feedings and had "distended abdomens and attacks of diarrhea with large, pale, foul smelling stools." Their stools also were found to have a low percentage of "split fats." The designation "CF of the pancreas" was derived from the finding that children who died in the neonatal period had characteristic histopathologic lesions of the pancreas. Most children survived the neonatal period, but in Anderson's series only 8 of 49 patients survived past their second birthday. In the latter patient group, 44 of 49 deaths were a result of bronchopulmonary infection usually due to *Staphylococcus aureus*. Two autopsy findings were characteristic of this disease. First, these patients had infections primarily in the airways rather than the lung parenchyma. The second important finding, confirmed by others (3, 60, 171, 210), was airway luminal plugging by "thick, tenacious, greenish gray, purulent material" (3).

Anderson's work described the two fundamental pathophysiologic problems encountered in CF: pancreatic insufficiency with malnutrition as well as airway infections. Both are related to the finding that these patients produce extremely viscous secretions. These secretions lead to autodigestion of the pancreas through blockage of pancreatic ducts and inability to secrete digestive enzymes. In the conducting airways the production of these secretions results in chronic infection due to several types of organisms. This review will

† This review was written in honor of Leon Croteau. Leon taught me the importance of optimism and good humor in life despite his losing battle with cystic fibrosis.

focus on these microbes and their epidemiology, pathogenic mechanisms, antimicrobial resistance, and laboratory detection in this patient population.

PATHOGENESIS OF CF

In her landmark study, Anderson realized that CF was likely a genetically based disease (3). The development of CF is now recognized as an autosomal recessive trait found in approximately 1 in 2,000 Caucasian children (144). The disease also occurs in Hispanic and black populations but at a much lower rate. In 1985, the gene thought to be responsible for CF was localized to a specific region on chromosome 7 (194, 198, 206). Using the technique of chromosome walking and jumping, a research team led by Collins, Riordan, and Tsui identified the actual CF gene on chromosome 7 (101, 158, 160). The specific gene product that is defective (158) is believed to be present in a transmembrane protein which these investigators have called CF conductance regulator. In 68% of the CF patients they have studied, a mutation has been detected in which a phenylalanine residue is deleted at amino acid position 508 in the CF conductance regulator (160). The mutation has been designated as $\Delta phe\text{-}508$. This deletion is thought to occur in a functionally important region of the protein and may adversely affect the regulation of ion transport at the apical surface of epithelial cells. This mutation is found primarily in patients with severe disease. Other mutations in this gene have been recognized but currently have not been as fully characterized. These uncharacterized mutations can result in either severe or mild disease. These findings, still in their infancy, are a major step towards understanding the pathophysiology of CF.

Even before the discovery of the CF gene and its putative product, investigators had begun to unravel the basic cellular defect found in CF. Knowles and colleagues (106) demonstrated that increased sodium absorption occurred across the respiratory epithelium of patients with CF. This defect was found to be stably expressed in tissue culture cells, and these cells have proven to be extremely valuable in further characterizing defects in ion transport (207, 212). Frizzell et al. (68), Widdicombe et al. (207), and Boucher et al. (20–22) have shown that there is a defect in the regulation of chloride ion secretion in CF respiratory epithelium as well. These observations may explain why patients with CF have comparatively dehydrated surface liquid on their respiratory epithelium (199). It is believed that this dehydration of respiratory secretions plays an important role in the defective mucociliary clearance seen in these patients.

Boat and colleagues (16, 36) reported that the proteoglycans in the mucus overlying the ciliated epithelium in CF patients have increased sulfation. These highly charged molecules may interact with bacteria or bacterial exoproducts in a manner that contributes to the viscosity of airway secretions. Together, the dehydrated surface liquid and increased sulfation of the airway glycocalyx may explain the development of tenacious secretions seen in the airways of CF patients. These secretions may provide a suitable environment for the growth of selected microbes.

AIRWAY INFECTIONS

The lungs of neonates with CF are histologically normal (3). However, in her initial report on CF done during the pre-antimicrobial era, Anderson clearly recognized the importance of airway infections in the abbreviated lifespan (usually <2 years) of CF patients. It is now recognized (62, 91, 144, 179, 190, 210) that pulmonary dysfunction is responsible for approximately 90% of deaths in CF patients and is largely a result of chronic airway infection. These chronic airway infections are characterized by intercurrent acute exacerbations with fever, weight loss, increased cough, change in volume, color, or appearance of sputum, increased respiratory rate, dyspnea, or rales and rhonchi on chest examination and appearance of new infiltrates on chest radiograph (131). Progressive deterioration in lung function as measured by pulmonary function testing is often the result (107, 127, 210).

A cycle of relative well-being complicated by repeated episodes of pulmonary dysfunction is common in CF patients. With each exacerbation, lung function declines, eventually leading to pulmonary failure and death. This process may take many years and, with effective new antimicrobial therapies, many patients with CF now survive into adulthood (127, 179, 210). A small number of patients remain relatively free of chronic lung infections and associated pulmonary disease. These patients fare quite well and have relatively long survival compared with the more typical individuals with CF who can now expect to live to their third or fourth decade (100, 127, 179, 210).

IMPORTANT PATHOGENS IN CF LUNG DISEASE

Chronic lung infection in patients with CF is usually associated with a limited number of organisms. Two bacterial pathogens, *S. aureus* and *Pseudomonas aeruginosa*, are most often recovered from the respiratory tract of chronically infected CF patients. In a 1986 survey of U.S. CF centers (100), *P. aeruginosa* was found in 60% of respiratory tract cultures, whereas *S. aureus* was found in 27%. Other organisms found with some degree of frequency in the respiratory tract of CF patients include *Haemophilus influenzae*, *P. cepacia*, and other glucose nonfermenters, including *Xanthomonas maltophilia*. In addition, the roles of *Legionella pneumophila*, *Mycobacterium* spp., viruses, and fungi, especially *Aspergillus* spp., in lung disease of CF patients are beginning to be more clearly understood.

Staphylococcus aureus

S. aureus was the first organism recognized to cause chronic lung infections in young CF patients. In the pre-antibiotic era, lung infection due to this organism was the leading cause of their mortality (4). It continues to be an important pulmonary pathogen, especially in CF patients who are <10 years old (12, 91, 96, 126, 179, 210, 211).

Virulence. The virulence of *S. aureus* is dependent on two factors: the ability to adhere to respiratory epithelium and, once bound, the ability to evade immune clearance. Two factors, teichoic acid (2) and slime (31), appear to play significant roles in adherence of *S. aureus* to respiratory epithelium in CF patients. How these two factors interact in this process is not known. Slime, produced by *S. aureus*, is biochemically distinct from expolysaccharides produced by *P. aeruginosa*. It is a loosely cell-associated, complex polysaccharide containing sugars, uronic acid, and amino acids (208).

Once bound, *S. aureus* produces a variety of virulence factors. Factors which may allow it to evade immune clearance include leucocidins, which can lyse phagocytic cells, capsules, and protein A. In addition, the organism produces a variety of other virulence factors, including hemolysins,

hyaluronidase, catalase, coagulase, and several exotoxins (39).

Precisely how *S. aureus* causes tissue damage in the lungs of CF patients has not been defined. Autopsy studies done during the pre-antibiotic era revealed that *S. aureus* infection caused significant lung damage which was responsible for the death of most CF patients (4, 213).

Antimicrobial therapy and susceptibility. Work on the pathogenesis of *S. aureus*-associated lung disease in patients with CF may have been somewhat limited because of the efficacy of antistaphylococcal therapy. Antistaphylococcal beta-lactams including nafcillin, oxacillin, and dicloxacillin have played a major role in controlling *S. aureus* infections in this population (12, 127, 182). In Europe, fusidic acid has also been used successfully to treat *S. aureus* infections (182). Long-term prophylaxis with trimethoprim-sulfamethoxazole (TMP-SMX), dicloxacillin, tetracyclines, and first-generation cephalosporins has some efficacy in suppressing *S. aureus* (12, 115, 127). Despite intensive antimicrobial pressure, resistance in *S. aureus* recovered from the respiratory tree of CF patients is usually limited to penicillin G. Methicillin-resistant *S. aureus* (MRSA) strains are infrequently recovered from this group of patients. Recently, Boxerbaum and colleagues (24) reported that, in one CF center, no MRSA was recovered in 1984 or 1985 but 14 of 212 patients had MRSA in 1986. They also reported that MRSA did not have a significant impact on the clinical course of CF patients even though these patients did not receive antimicrobial agents specifically targeted for the MRSA. In addition, 10 of 14 CF patients spontaneously lost the organism, whereas colonization persisted in 4 patients. This report suggests, and our experience would confirm, that MRSA is not of concern in the CF population.

Antimicrobial prophylaxis. Two specific problems are associated with long-term antistaphylococcal prophylaxis. First, some data suggest that *P. aeruginosa* colonization or infection has emerged more rapidly in patients receiving antistaphylococcal prophylaxis. Unfortunately, these data are based primarily on clinical impressions and retrospective analyses (11, 12, 70, 108). One of the few placebo-controlled trials looking at efficacy of antistaphylococcal prophylaxis included only 17 patients, 11 of whom were colonized with *P. aeruginosa* on entry into the study; therefore, it is difficult to assess adequately the role of antistaphylococcal therapy in promoting *P. aeruginosa* infection in these patients (115). A placebo-controlled trial sponsored by the Cystic Fibrosis Foundation is being performed to determine the efficacy of long-term antistaphylococcal prophylaxis in CF patients.

Second, an example of antimicrobial pressure resulting in *S. aureus* resistance has been found in patients receiving long-term TMP-SMX prophylaxis. Several investigators (75, 95, 172) have noted that TMP-SMX resistance in this situation is due to the organism's ability to take up thymidine from its environment rather than synthesize it by a pathway that is inhibited by TMP-SMX. Strains that can take up thymidine from their environment are designated as being thymidine dependent. We found that, among 95 patients with *S. aureus*, 20 had thymidine-dependent *S. aureus* strains (TDSA) (75). Of 39 patients with *S. aureus* who had received TMP-SMX prophylaxis, 20 harbored TDSA. The mean duration of TMP-SMX therapy in this group was 30.9 months compared with 11.0 months in 19 patients with no TDSA strains. The clinical significance of TDSA is not known. It is known that TDSA has abnormal colonial morphology or does not grow at all on most commonly used isolation media. However, TDSA has typical colonial morphology on mannitol-salt agar (75). Thus, to ensure consistent recovery of all *S. aureus* isolates, we recommend that this medium be used for all respiratory tract specimens from CF patients.

Pseudomonas aeruginosa

With the advent of effective antistaphylococcal antimicrobial therapy, *P. aeruginosa* emerged as the most important bacterial pathogen in lung disease of CF patients (10, 25, 62, 91, 96, 108, 126, 179, 190). A unique feature of CF patients chronically infected with *P. aeruginosa* is the recovery of an unusual morphotype from respiratory secretions. This observation was first made by Doggett et al. (54, 55) and since has been confirmed by others (10, 25, 62, 91, 96, 108, 126, 179, 190). This morphotype was designated "mucoid" by Wabha and Darrell, who have described six *P. aeruginosa* morphotypes (197). All six morphotypes have been found in sputum specimens from CF patients (189). For any given patient early in infection, the classical morphotype appears to predominate (91, 140, 157, 210). However, mucoid *P. aeruginosa* soon becomes the most common morphotype (81, 140, 144), although other morphotypes may frequently be recovered concurrently. In two different populations of CF patients, one in Cleveland, Ohio (189), and the other in Denmark (90, 91), approximately 80% of *P. aeruginosa* isolates were mucoid. In contrast, in a study of non-CF patients, only 3% of *P. aeruginosa* respiratory tract isolates were mucoid (90).

The mucoid morphotype is due to the production of large amounts of polysaccharide that surround the cell. This material has been designated by Pier (144) as "mucoid exopolysaccharide," or MEP. In earlier studies, this material was referred to as "slime" by Evans and Linker (61). Chakrabarty and his co-workers (48) have designated this material as "mucoidy." The material to which these studies refer is a polymer composed of acetylated D-mannuronic acid and L-guluronic acid and is commonly called alginate (8, 9, 43, 66, 67, 134, 161). In this review, Pier's term, MEP, will be used. Pier et al. (145), using immunochemical techniques, demonstrated that all strains of *P. aeruginosa* isolated from their CF patients produced MEP, even those classified as nonmucoid morphotypes.

Virulence. *P. aeruginosa* isolates produce numerous, diverse virulence factors including exotoxin A, exoenzyme S, elastase, alkaline protease, pyoverdin, two types of hemolysins, lipopolysaccharide, pili, and MEP (58, 62, 94, 130, 190). Literature covering these factors is exceedingly broad and complex. Only selected aspects of this literature can be reviewed here. Those interested in further reviewing this topic should consult two excellent volumes on *P. aeruginosa* virulence: one edited by Hoiby and colleagues (94), and the other edited by Doring et al. (58). Because of the ubiquity of the mucoid phenotype in the lung of older CF patients, the role of mucoid *P. aeruginosa* in the pathogenesis of lung disease will be briefly reviewed.

Role of mucoid *P. aeruginosa* in pathogenesis of CF lung disease. The initiating events in the establishment of chronic lung infection with mucoid *P. aeruginosa* are not clearly understood, nor have any long-term longitudinal studies been done to clarify this issue. Initial colonization of the airways is usually due to nonmucoid isolates (91, 140, 157, 210). Under some undefined environmental pressure, these organisms convert to the mucoid phenotype, and it predominates during chronic lung infection in these patients (15, 48, 52, 80, 155, 174). Costerton and colleagues (42, 110) have

speculated that mucoid *P. aeruginosa* grows as microcolonies in the lung and that microcolony formation plays an important role in the pathogenesis of this infection. Microcolony formation may be enhanced by proteases locally produced by *P. aeruginosa*. Klinger et al. (103) reported that pseudomonal proteases cause release of mucins from the respiratory epithelium. Mucins in CF patients are highly sulfonated (16, 36) and, when combined with MEP in the presence of calcium ions, form a highly viscous gel (82). Increased gel formation enhances microcolony formation, which may lead to even poorer ciliary clearance of these organisms.

Not only is ciliary clearance of mucoid *P. aeruginosa* probably limited, but also the organism may evade phagocytosis. MEP has been shown to be antiphagocytic in vitro, and optimal killing of *P. aeruginosa* by phagocytes requires opsonic antibodies (6, 147, 173). Several pieces of evidence suggest that opsonization of mucoid *P. aeruginosa* is limited in vivo, thus enhancing the ability of mucoid *P. aeruginosa* to evade immune clearance in the lung.

Several investigators have found that serum from CF patients inhibits phagocytosis (93, 188). This inhibitory activity is believed to be due to specific antipseudomonal antibodies, primarily of the immunoglobulin G2 (IgG2) class, which do not bind to receptor on alveolar macrophages and are thus not opsonic (64). Hoiby and Olling (93) have found that some CF patients possess antibodies that block serum bactericidal activity against *P. aeruginosa*. Since IgG is the major immunoglobulin and, thus, opsonin in bronchial fluid (56), it would be reasonable to expect to see these same effects due to IgG in the lung.

Another observation that may explain the poor opsonization of mucoid *P. aeruginosa* in the CF lung was made by Fick and colleagues (63, 65). They have shown that pseudomonal proteases degrade >80% of IgG in bronchial fluid, resulting in the inhibition of *P. aeruginosa* killing by pulmonary macrophages in vitro. Their explanation for this finding is that degradation of IgG occurs in a manner such that the F_{ab} portion remains intact. The F_{ab} portion binds to the organism, but because the F_c portion is absent, binding to the phagocytic cells and thus opsonization do not occur.

The long-term survival of mucoid *P. aeruginosa* in the lung may lead to damage to the lung in two ways. First, tissue damage due to the in situ production of pseudomonal virulence factors such as elastase, exoenzyme S, and exotoxin A may occur. Second, the immune response to this chronic infection may be responsible for tissue damage as well.

CF patients with high levels of circulating immune complexes have poorer lung function than those with lower levels (153, 161). Bronchial fluids from CF patients infected with mucoid *P. aeruginosa* have been shown to contain immune complexes, and these patients have increased inflammatory reactions in the lung (56, 57). It would appear that the inflammatory response to the presence of immune complexes in the lung may be responsible, in part, for the deterioration of lung function seen in these patients.

Besides immune complex-mediated damage to the lung, Suter and colleagues have demonstrated that there are very high levels of elastase and protease of phagocytic cell origin in the lungs of CF patients infected with *P. aeruginosa* (180, 181). Although antiprotease inhibitors are present in airways, it is thought that the levels of protease released from the phagocytes are far in excess of the amount that the inhibitors can inactivate (180, 181). The observation that CF patients with more advanced airway disease have high levels of granulocyte elastase activity in their bronchial secretion than patients with early disease (29, 181) supports this idea. At autopsy, the lungs of three of three patients showed altered lung elastin, indicating that proteolytic enzymes of both phagocytic and bacterial origin may be important mediators of airway destruction that occurs during chronic *P. aeruginosa* lung infection (29).

These data indicate that the immune response to *P. aeruginosa* makes a significant contribution to the tissue damage seen in the lungs of infected CF patients. One of the treatment strategies under long-term clinical trial is the use of corticosteroids to prevent or reduce tissue damage in the lungs due to inflammation. In a small number of CF patients, Auerbach et al. (5) have shown that prednisone administered on alternate days reduces morbidity and improves pulmonary function in patients with mild to moderate lung disease. Prednisone appears to have two beneficial effects. One effect is the reduction in serum IgG levels, which correlates with improved lung function and probably reflects decreased inflammation and immune complex formation in the lung. Second, corticosteroids may promote morphologic differentiation of nonciliated epithelial cells to ciliated cells, thus enhancing pulmonary clearance (22). The use of nonsteroidal anti-inflammatory agents such as ibuprofen is being considered for study as well.

Regulation of MEP production. Although the mechanism by which mucoid *P. aeruginosa* contributes to lung disease in CF patients is being elucidated, factors involved in the expression of the mucoid phenotype in the CF lung are only now beginning to be understood. The conventional wisdom of pseudomonal infection in CF patients is that initial infection is with nonmucoid *P. aeruginosa* and that mucoid strains emerge and become predominant as the infection becomes chronic. As mentioned, all *P. aeruginosa* isolates recovered from CF patients studied by Pier et al., regardless of colonial morphotype (145), can produce MEP. Many CF patients harbor both mucoid and nonmucoid strains concurrently. Only recently has the genetic control of the switching between nonmucoid and mucoid phenotypes begun to be understood.

Early studies showed that nonmucoid isolates could be converted to mucoid ones in the presence of carbenicillin, certain phages, and pyocins (82, 135, 161). Recent work from Chakrabarty and colleagues has revealed the events that may lead to conversion of nonmucoid to mucoid strains. In a series of publications (8, 9, 15, 43–45, 48–52, 73, 133, 156, 162, 201), they have demonstrated that a cluster of genes on the *P. aeruginosa* chromosome is responsible for the initial steps in MEP synthesis. The expression of these genes is under regulatory control by a gene which they designate *algR* (43, 45, 48). Mutations in this gene or in the gene cluster lead to the loss of the ability to produce MEP. In addition, by using complementation experiments in which DNA sequences are reintroduced into the chromosome at the site of these mutations, they produced clones that regained the ability to produce MEP (43–45, 49–51, 73, 162, 201).

Flynn and Ohman (66, 67) and MacGeorge et al. (118) have identified another chromosomal region which also appears to be involved in regulating the conversion of nonmucoid to mucoid strains. Flynn and Ohman (66, 67) have designated this region *algST*, whereas MacGeorge et al. (118) have designated it MUC. These regions are found in similar locations on the *P. aeruginosa* chromosome. Flynn and Ohman (67) have suggested that the *algS* gene controls expression of the *algT* gene and that the *algT* gene product regulates alginate synthesis. Whether the *algT* product acts

directly on the *algR* gene or directly on the cluster of the alginate synthetic genes is not known. The precise mechanism for the genetic regulation of alginate synthesis remains to be elucidated.

It has long been speculated that the CF lung is a unique biologic environment and that unknown factors in this environment lead to the expression of the mucoid phenotype (35, 135). Now that the genetic events necessary for the expression of this phenotype are beginning to be understood, it will be possible to design experiments to determine which factors regulate gene expression. Preliminary experiments have been reported in which conditions of high osmolarity lead to increased expression of the *algD* gene, one of the genes in the gene cluster responsible for MEP production (15, 48, 52). Speert et al. (174) used extreme nutritional stress over extended incubation times (a minimum of 30 days) to attempt to induce the *P. aeruginosa* mucoid phenotype. They found that mucoid variants could be recovered from 20 of 104 (19.2%) nonmucoid isolates exposed to these conditions. The rate at which mucoid variants arose was similar for environmental (24.6%), CF (14.3%), and non-CF (12.1%) clinical isolates. These data suggest that the *P. aeruginosa* strains that infect the airways of CF patients are not necessarily unique, but rather the environment of the CF lung contains factors which stimulate the expression of the mucoid phenotype.

Mucoid *P. aeruginosa* strains have two phenotypic characteristics that differ from classical strains. Mucoid strains tend to be serum sensitive (86, 140, 163, 164). By the International Antigenic Typing System, which is based on the O-polysaccharide component of lipopolysaccharide (28), mucoid strains are usually polyagglutinable. Classical strains are serum resistant and usually monoagglutinable (86, 140). The polyagglutinability is probably due to a lack of or reduced number of polysaccharide side chains in these lipopolysaccharides (69, 88, 181). What role these phenotypic factors play in the pathogenesis of infection with this organism is not known.

Immunity to mucoid *P. aeruginosa*. Some CF patients survive to adulthood without becoming chronically colonized or infected with mucoid *P. aeruginosa*. Pier and colleagues (147) have reported that these patients had "opsonophagocytic" antibodies that were specific for MEP. Colonized patients also had opsonophagocytic antibodies but they were not specific for MEP. These MEP-specific antibodies were found in only 1 of 10 noncolonized young CF patients and not in normal controls. These data suggest that, if an immune response could be mounted which was specific for MEP, CF patients would likely be protected from *P. aeruginosa* infection. This observation has important implications for vaccine development against this organism (146, 147). A vaccine made of a glycine-EDTA extract of *P. aeruginosa* whole cells and a heptavalent lipopolysaccharide pseudomonal vaccine have been developed. Neither proved to be of value in protecting CF patients against pseudomonal infection (112, 128, 141). Vaccines designed to elicit opsonophagocytic antibodies to MEP may prove to be useful in protecting against infection with mucoid *P. aeruginosa*.

Antimicrobial resistance and susceptibility testing. A comprehensive discussion of the development of antimicrobial resistance in *P. aeruginosa* lung infection is too complex for this review. However, several points should be made concerning antimicrobial therapy in CF patients. The issue of whether *P. aeruginosa* can be eradicated from the lungs of CF patients by antimicrobial agents is an important one. When quantitative sputum cultures are done on CF patients treated with antipseudomonal therapy, the posttreatment quantity of *P. aeruginosa* often declines dramatically and, in some patients, the organism may become undetectable (70, 107, 127, 138, 152, 169). However, in most patients, 1 to 3 months following treatment, the quantity of *P. aeruginosa* in sputum returns to pretreatment levels. Whether the reappearance of *P. aeruginosa* after antimicrobial therapy (as it almost always does), represents infection with a new strain or failure to clear the preexisting strain is not known (99). Until recently, epidemiologic markers used to address this issue, such as serotyping, phage typing, phenotyping, pyocin typing, and antibiograms, have been imprecise (27, 149). Ogle and colleagues (132, 133) have developed a molecular probe that has allowed for more precise studies of the epidemiology of *P. aeruginosa*. Preliminary work with this probe suggests that genotypes persist during and after antimicrobial therapy and that more than one genotype can be present in the lungs at the same time. In addition, genotypes can also change over time. Longitudinal studies done over extended periods of time with this technique are needed to improve our understanding of the epidemiology of infection with this organism.

Because of relatively poor penetration of a number of antimicrobial agents into the respiratory tract of CF patients (14, 113, 179), the antimicrobial concentrations at that site are often subinhibitory for *P. aeruginosa*. The effect of these subinhibitory concentrations on the virulence of *P. aeruginosa* is beginning to be more clearly understood and may explain at least one observation made concerning the efficacy of therapy. Clinical improvement during antimicrobial therapy is seen in CF patients who have either no reduction or only a modest reduction in the number of organisms as measured by quantitative culture. This improvement is often attributed to adjunctive therapies such as improved hydration, oxygenation, and lung physiotherapy (70, 109, 120, 131, 157, 179, 199), and these factors clearly are important in the comprehensive care of the CF patient. However, subinhibitory concentration of several antimicrobial agents have been shown to inhibit the expression of a number of *P. aeruginosa* virulence factors in vitro.

Vishwanath et al. (195) showed that subinhibitory concentrations of ceftazidime inhibit the binding of mucoid *P. aeruginosa* strains to tracheobronchial mucins but tobramycin does not. Geers and Baker (71, 72) found that MEP production is greatly reduced in the presence of aminoglycosides and, as a result, binding to epithelial cells is also reduced. Their observations appear to conflict with those of Vishwanath et al. However, comparisons of these two studies are difficult because adherence was measured by using two different experimental systems. Geers and Baker (71, 72) also found that subinhibitory concentrations of aminoglycosides inhibited exotoxin A and elastase production in both broth and organ culture whereas cephalosporins did not. Grimwood et al. (83) demonstrated that tobramycin inhibited elastase production in vitro but did not find significant reduction in exotoxin A production. Subinhibitory concentrations of ciprofloxacin reduced in vitro production of exotoxin A, exoenzyme S, and elastase, while ceftazidime reduced elastase and phospholipase C levels. In a rat model of pneumonia, both tobramycin and ciprofloxacin inhibited exoenzyme S and elastase production, whereas ceftazidime inhibited only exoenzyme S production. Histologic preparation of lung sections demonstrated much less tissue damage in animals treated with agents that inhibit elastase production. These data support the hypothesis that localized production of elastase is an important initiating event in the

progressive lung disease seen in CF. Clinical improvement in the presence of subinhibitory concentrations of antimicrobial agents may be explained by suppression of virulence factors that are responsible for the pathogenicity of these organisms in the lung.

There are two widely different philosophies about performing susceptibility testing of *P. aeruginosa* isolates. Multiple morphotypes (as many as six in our experience) of *P. aeruginosa* may be recovered in a single sputum specimen. One approach is to perform a separate susceptibility test on each morphotype. An alternative approach is to test all morphotypes together in one susceptibility assay (122). Thomassen et al. (189) reported that different morphotypes from the same patient had different susceptibilities 51% of the time, but that similar morphotypes gave similar results >85% of the time. Govan et al. (80) pointed out that organisms of the same morphotype could have very different antimicrobial susceptibility patterns and that susceptibility of the isolates could change rapidly over the course of antimicrobial therapy. This group advocated testing as many as 20 "colonial representatives" by using agar dilution testing, something that clearly is not practical in most clinical laboratory settings. Testing different morphotypes is time-consuming and expensive. It remains to be determined whether the data generated are of value or whether a technique testing all morphotypes together will give similar results at less cost. Maybury et al. (122) found that, when mixed morphotypes were tested, 94% of MIC results were within ±1 dilution of the most resistant morphotype when each morphotype was tested individually for all antibiotics. These data suggest that testing multiple morphotypes together is an accurate and inexpensive alternative to testing each morphotype individually.

Antimicrobial pressure often results in significant changes in susceptibility of *P. aeruginosa* to the agent that is being used therapeutically (18, 77, 139, 142, 167, 168, 202). Several studies have shown that increases in MICs, some dramatic, often occur during therapy with a variety of antimicrobial agents (26, 46, 77, 89, 139, 142, 167, 168, 176). Not surprisingly, it appears that a resistant subpopulation is selected that can persist in the presence of a specific antimicrobial agent. Shalit et al. (168), using quantitative culture techniques, found that after 2 weeks of therapy with ciprofloxacin approximately 60% of patients harbored ciprofloxacin-resistant *P. aeruginosa*. Usually only a small percentage (0.01 to 10%) of the total *P. aeruginosa* population was resistant.

Two other points must be considered when examining the issue of antimicrobial pressure. First, when antimicrobial therapy is discontinued, populations of *P. aeruginosa* from these patients often, but not always, regain susceptibility to the particular antimicrobial agent used. Second, resistant organisms that emerge due to antimicrobial pressure may persist and be spread to other CF patients in the treatment center (139). It is not clear whether antimicrobial pressure has any role in this spread or persistence of these organisms, but it is clear that *P. aeruginosa* is extremely adaptable to interactions with various antimicrobial agents and resistant subpopulations frequently emerge. Therefore, *P. aeruginosa* susceptibility must be carefully monitored to assist the clinician in making appropriate therapeutic choices.

Pseudomonas cepacia

Prevalence. During the early 1980s, *P. cepacia* emerged as a pathogen of potential importance in the lung disease of patients with CF. This organism has been associated with significant mortality at CF centers in Philadelphia, Pa.; Cleveland, Ohio; and Toronto, Ontario, Canada (97, 184, 185, 192). At these three centers, as many as 20% of patients, primarily young adults, were colonized with this organism. The disease followed one of three clinical courses. In some CF patients, long-term colonization occurred without adversely affecting lung function. In others, chronic infection associated with slowly declining lung function was seen. Finally, in another group of patients, acute fulminant lung infection, leading to death in weeks to months, was observed (97, 192).

Because of the apparent devastating nature of this infection in a subpopulation of CF patients, the Cystic Fibrosis Foundation has initiated a nationwide study to determine the magnitude of the problem. Preliminary results (185a) indicate that this organism is not as widely prevalent in most CF centers as it was in the three centers that first reported it. In a multicenter study that did not include the Toronto, Cleveland, or Philadelphia centers, Welch and colleagues (205) found the prevalence to vary from 0 to 14% of patients at seven geographically diverse centers and the overall mean isolation rate to be 6.1%.

Laboratory detection. One of the difficulties in attempting to determine the importance of this organism is that, until the development of selective media for its isolation, *P. cepacia*, in all likelihood, was extremely difficult to recover from respiratory secretions, especially in patients heavily colonized with mucoid *P. aeruginosa* (74). MacConkey agar was the standard medium used to attempt to grow this organism. Recently, two agar media, PC (74) and OFPBL (205), have been developed for the specific isolation of *P. cepacia*. In a laboratory proficiency test exercise done by the Centers for Disease Control before the launch of the Cystic Fibrosis Foundation's national survey, only 36 of 115 laboratories isolated *P. cepacia* from mock sputum specimens (183). By using one of the two *P. cepacia* selective media, 14 of 15 laboratories isolated this organism, whereas only 22 of 100 that used primarily MacConkey agar as their isolation medium were successful. The laboratories that failed the initial test were asked to repeat it with *P. cepacia* selective media. Seventy-five laboratories agreed to participate, and 73 of 75 successfully isolated *P. cepacia*. In a multicenter study with 725 clinical specimens (205), *P. cepacia* was isolated from 58 specimens with OFPBL medium and from only 19 specimens with MacConkey agar. In a different study, PC medium was compared with MacConkey agar for detection of *P. cepacia* from 169 clinical specimens. Of 35 isolates recovered (74), all 35 were found on PC medium, whereas only 21 were recovered on MacConkey agar. Two factors contribute to the superiority of PC and OFPBL media: (i) growth of *P. cepacia* frequently is obscured by mucoid *P. aeruginosa* on MacConkey agar, whereas the growth of *P. aeruginosa* isolates is usually inhibited on the two selective media; and (ii) *P. cepacia* grows more rapidly on these media than on MacConkey agar. Colonies have been observed after 24 to 48 h of incubation on PC and OFPBL media in contrast to 72 h for visible colonies on MacConkey agar (74, 205).

PC and OFPBL each have specific advantages and disadvantages. PC medium is more complex to prepare and is selective only, whereas OFPBL is both differential and selective (74, 205). However PC is more selective than OFPBL and is more effective in supporting the growth of *P. cepacia* (33). In addition, another *Pseudomonas* sp., *P. gladioli*, has been recovered from sputa of CF patients with OFPBL. This organism resembles *P. cepacia* on OFPBL

(205) and has been confused with it. Christenson et al. (38) evaluated three patients colonized with *P. gladioli* and found that the organism did not appear to have a significant role in their lung disease. In summary, both OFPBL and PC media are superior for the recovery of *P. cepacia* from sputum. The choice between these two media should be based on individual laboratory experience.

Epidemiology. Factors that lead to colonization or infection by *P. cepacia* are just beginning to be understood. Tablan and colleagues (184, 185) did retrospective case control studies at two different CF centers trying to understand the epidemiology of *P. cepacia* infection in CF patients. They found that the risk for *P. cepacia* colonization or infection was increased by (i) the severity of underlying pulmonary disease, (ii) having a sibling with CF who was also colonized, (iii) increasing age, and (iv) hospitalization in the previous 6 months. In one of the two centers, the use of aminoglycoside antimicrobial agents was also associated with increased risk for *P. cepacia* infection (184). Because siblings were at increased risk for colonization, person-to-person transmission was suggested. Thomassen et al. (191) have reported that colonization or infection rates in their institution declined when colonized patients were isolated from noncolonized patients. In the year before isolation precautions were instituted, the incidence of *P. cepacia* was 8.2%. After institution of these infection control measures, the incidence dropped to 1.7%. These data further support the role of person-to-person spread.

Alternatively, patients may acquire this organism from other sources in their environment. *P. cepacia* infections associated with environmental contamination of disinfectants and distilled water have been well characterized in non-CF patients (137). Environmental sampling in two CF centers revealed that the organism was infrequently isolated. At one center, 3 of 141 cultures were positive. Surface cultures from all respiratory therapy and pulmonary function test equipment were negative (87). At the other center, a positive culture was obtained from equipment used to test pulmonary function (97), suggesting a possible source. In a study of 35 patients, this organism was not recovered from their home aerosol therapy equipment even though 21 patients using it were colonized with *P. cepacia* (148). In contrast, *P. aeruginosa* was recovered from the respiratory therapy equipment of 5 of 36 (31 colonized) patients. These data suggest that this equipment is more likely to be contaminated by *P. aeruginosa* than by *P. cepacia*. Much more information is needed before the way in which *P. cepacia* enters and spreads through a population of CF patients is understood.

Typing techniques. One of the difficulties with attempting to study the epidemiology of infection with *P. cepacia* is the paucity of tools available for classifying these isolates. Serotyping (88), biotyping (78), and bacteriocin typing (82) have all been described for *P. cepacia*. In hospital outbreaks that were probably due to a single source, serotyping was found to be of value (88). However, in CF patients, 40% of isolates either agglutinated in multiple typing sera or were nonagglutinable, suggesting that serotyping is probably of little value in this population (78). Biotyping schemes have also been developed. Although simple to do, biotyping is probably of limited value in epidemiologic studies. Govan et al. (82) have used bacteriocin typing and production for strain discrimination. Fifteen of the isolates tested were obtained from J. Klinger, Rainbow Babies and Childrens Hospital, Cleveland, Ohio, which is a center with a high incidence of *P. cepacia*-infected patients. These isolates proved to be a bacteriocin type that was uncommonly recovered at other hospitals. Unfortunately, it was not reported whether these isolates were from CF patients or not. The bacteriocin types appeared to be relatively stable when isolates were frozen at −70°C or kept on minimal medium at room temperature. This technique is laborious and depends on the continued production of bacteriocins by the producer strains.

A technique that may greatly aid in studying the epidemiology of *P. cepacia* in CF patients is ribotyping. This technique, used by LiPuma, Stull, and colleagues (114, 177) depends on rRNA probing of restriction endonuclease digests of chromosomal DNA of the target organism. In initial studies (177), they found that the same ribotype persisted over time in individual patients and that siblings usually had the same ribotype. Further studies (114) revealed that a specific ribotype predominated in each of three CF centers from which isolates were obtained for study. The ribotype of the predominant strain at each center was different, suggesting that each center had its own "resident" strains. If ribotyping proves to be a sensitive and specific discriminator of genotype in *P. cepacia*, it may be possible, using this technique, to begin to understand the epidemiology of infection with this organism.

Pathogenic potential. One of the great paradoxes concerning *P. cepacia* is whether this organism is a true pathogen or just a marker of severe lung disease. In the two case-controlled studies discussed above (184, 185), the patients who had *P. cepacia* tended to have much poorer pulmonary status than those without infection. Several possibilities exist to explain why *P. cepacia* infection is associated with a spectrum of disease from asymptomatic carriage to acute, fulminant, fatal infection (97, 184, 185). First, *P. cepacia* strains may vary significantly in virulence from very low to extremely high levels. Second, it may act synergistically with other unidentified agents to cause fulminant infection in a manner similar to that seen with *S. aureus* and influenza virus (32). Third, the severity of *P. cepacia* infection may be dependent on tissue damage caused by other organisms. Severe, preexisting tissue damage may be required before *P. cepacia* can initiate a fatal infection. Alternatively, instead of playing an active role in the patient's lung disease, *P. cepacia* may act as a marker for severe irreparable tissue damage. Whatever the role of *P. cepacia* in lung disease in CF patients, the organism is essentially impossible to eradicate from the lung, probably due to its resistance to multiple antimicrobial agents (76, 78, 152, 192). It is not clear whether *P. cepacia* plays an active role in tissue destruction, or whether it colonizes lungs that are so ravaged by infections by other organisms that this tissue can no longer resist *P. cepacia* colonization.

Data are available that would argue that *P. cepacia* is nothing more than a colonizer. This organism is essentially avirulent in animal models when compared with *P. aeruginosa*. In a guinea pig model of pneumonia, *P. cepacia* caused only mild lung disease, while similar concentrations of *P. aeruginosa* caused a fulminant fatal pneumonia (78). Goldmann and Klinger (78) have shown that *P. cepacia* can persist for at least 2 weeks in the rat model of chronic lung infection described by Cash et al. (34). No animals in their studies died, although pathologic changes were seen in the lung. Stover et al. (175), using a burned mouse model of infection, showed that compared with *P. aeruginosa*, which was highly virulent in this model, *P. cepacia* was avirulent except at extremely high levels (10^5 CFU/ml), although it could persist in cutaneous burn wounds for at least 3 weeks.

TABLE 1. Virulence factor production of *P. cepacia* isolates

Test	No. positive/no. tested	
	Gilligan et al. (reference 75a)	McKevitt and Woods (reference 89)
Casein hydrolysis	65/85	44/48
Gelatinase	57/85	36/48
Elastase	0/85	0/48
Lipase	85/85[a]	33/48[b]
Hemolysin	0/85	1/48
Esterase (C4)[c]	30/30	
Esterase lipase (C8)	30/30	

[a] Hydrolysis on Tween 20 agar.
[b] Hydrolysis on egg yolk agar.
[c] Determined with the API ZYM test system (Analytab Products, Plainview, N.Y.)

Perhaps the data are most consistent with the observation that patients with severely damaged lungs are colonized but the organism has only a limited or no role in the progressive decline in lung function.

Alternatively, *P. cepacia* strains may vary in virulence, with certain isolates being highly virulent while others are essentially avirulent. *P. cepacia* strains recovered from CF patients have been studied to determine what virulence factors the organism might produce. McKevitt and Woods (125) studied 48 isolates of *P. cepacia* recovered from CF patients. They found that most of these isolates degraded casein and gelatin and were lipolytic (Table 1). Their group also found that protease purified from a clinical isolate when instilled in a rat lung could induce bronchopneumonia (124). However, none of the isolates produced elastase, exoenzyme S, or toxin A, all important *P. aeruginosa* virulence factors. They also found that culture supernatants had no activity against three different cell culture lines.

We examined the proteolytic and lipolytic activity of *P. cepacia* isolates recovered from CF patients (Table 1) (75a). Among our isolates, we found rates of proteolytic activity similar to those seen by McKevitt and Woods (125). We also found that all isolates had lipolytic activity, although direct comparison of our data to those of McKevitt and Woods is not possible because Lonon et al. (117) have reported that lipase activity of *P. cepacia* on Tween 20 does not correlate with activity on egg yolk agar. We also found that *P. cepacia* was extremely active against short-chain fatty acids, an observation that has alsc been reported by Poh and Loh (150). Preliminary studies of purified lipase by Lonon et al. (117) indicated that this enzyme is not cytotoxic to HeLa cells nor is it toxic when injected into mice. It is difficult to reconcile clinical observations which suggest that *P. cepacia* is highly virulent, at least in some patients, with in vitro and animal studies of virulence factors which indicate that *P. cepacia* is relatively avirulent. Much more work is needed to determine the exact role of *P. cepacia* in lung disease in patients with CF.

Antimicrobial resistance. Antimicrobial resistance clearly plays an important role in the ability of *P. cepacia* to persist in the lungs of CF patients. The organism is usually resistant to the aminoglycosides, colistin, carbenicillin, ticarcillin, and imipenem (77, 97, 152, 192). Although on initial isolation it is often susceptible to the ureidopenicillins and ceftazidime, the organism possesses an inducible beta-lactamase (37) and resistance due to derepression of this enzyme may be a problem posttherapeutically. The quinolones are not active against *P. cepacia* at concentrations achievable in lungs (178, 204), indicating that these antimicrobial agents will not be useful therapeutically. On initial isolation, many isolates are susceptible to chloramphenicol and TMP-SMX; however, resistance to these agents develops during or after therapy. Some isolates may be resistant to all available antimicrobial agents, a problem also seen occasionally with *P. aeruginosa*. Antimicrobial therapy rarely, if ever, leads to eradication of *P. cepacia*, and this organism has been seen to emerge during therapy for *P. aeruginosa*.

A novel approach for treating *P. cepacia* infection is now being explored. The studies involve aerosolized amiloride, a diuretic, that is being used experimentally in the therapy of adult CF patients (105). The rationale for the use of amiloride in patients with CF is as follows. One of the major cellular defects in CF patients is excessive reabsorption of sodium (Na^+) across epithelium cell membranes (106, 199). The reabsorption of Na^+ into airway epithelial cells is accompanied by reabsorption of water from the lung surface liquid, causing this liquid to become "dehydrated" which may explain, in part, the viscous secretions seen in CF patients. Amiloride, when aerosolized into the lung, blocks Na^+ reabsorption (199) and, thus, should prevent dehydration, resulting in less viscous secretions in CF patients. Guinta and colleagues reported that, in addition to its diuretic effect, this compound has antimicrobial properties (84). Work by Cohn and colleagues (40) showed that amiloride and tobramycin act synergistically against *P. cepacia* at concentrations achievable in the lung by aerosolization. Aerosolization of amiloride with tobramycin may prove to be a novel therapeutic strategy to treat *P. cepacia* infection.

Other Bacterial Agents

Haemophilus influenzae. During the 1986 survey of CF centers (100), *H. influenzae* was recovered from 11% of cultures done on respiratory secretions of CF patients. *H. influenzae* was recovered from 12% of patients at a German CF center (13) and from 14% at a Danish CF center (91). *H. influenzae* is primarily, but not exclusively, found in young children with CF. The organism is usually nontypable (116), which is characteristic of the isolates found in patients with other chronic respiratory diseases. The isolates are most frequently biotype 1 (116, 203), a trait that has been associated with enhanced virulence (116). Beta-lactamase-producing isolates have been found in up to 20% of CF patients (116, 123, 151). The percentage of isolates that are beta-lactamase positive varies from center to center, probably reflecting the known variation found in each geographic locale (53) rather than any unique feature of *H. influenzae* recovered from CF patients. Unlike *S. aureus*, *P. cepacia*, and *P. aeruginosa*, *H. influenzae* does not persist for extended periods of time in the lung (10). Although it has been associated with pulmonary exacerbations in CF patients (151), there is no evidence to suggest that it has a primary role in their chronic progressive pulmonary decline.

Isolation of hemophili from the respiratory secretions of CF patients can be challenging, especially in patients coinfected with mucoid *P. aeruginosa*. Roberts and Cole (159) used a hemin-bacitracin medium for isolation of *H. influenzae*. One of the key factors for successful isolation was to incubate the plates anaerobically. Anaerobic incubation suppressed the growth of *P. aeruginosa* while allowing the growth of hemophili. Wong et al. used quantitative culture techniques and selective agar (209). They found that *H. influenzae* was reliably recovered with this method, although how well this approach compared with qualitative

cultures was not clear. Bauernfeind et al. (13) used pyocins to inhibit the growth of *P. aeruginosa* in order to enhance *H. influenzae* recovery. They found that 6 of 17 *H. influenzae* isolates would not have been detected if this technique had not been used. They did not test anaerobic cultures in parallel; therefore, it is difficult to know whether the added work necessary to perform cultures with pyocin is of value.

Terpstra et al. (187) have developed a genomic DNA probe that hybridizes with *H. influenzae* as well as *H. parainfluenzae*, *H. parahaemolyticus*, and *H. haemolyticus*. In situ hybridization studies were positive with seven sputum smears from CF patients, while cultures for *H. influenzae* were positive in three of these seven probe-positive specimens. The authors claimed that "false-negative" cultures for *H. influenzae* occurred in the remaining four specimens. However, an alternative explanation may be that other hemophili were present, giving rise to a false-positive hybridization test. Since fairly simple culture techniques exist and the hybridization tests described are labor intensive, this approach does not seem practical even if the specificity of the test could be improved.

***Streptococcus pneumoniae*, enterics, and other glucose nonfermenters.** *Streptococcus pneumoniae* (92), enteric bacilli (96, 126), and gram-negative glucose-nonfermenting bacilli other than *P. aeruginosa* and *P. cepacia* (7, 104) are occasionally recovered from the respiratory secretions of CF patients. Like *H. influenzae*, none of these organisms seems to persist for extended periods of time (10, 91). Any role that they may have in lung disease of these patients is likely to be secondary to that of the organisms discussed previously.

Mycobacteria. The role of mycobacteria in chronic lung disease of CF patients has not been extensively studied. Wood et al. (210) found only two cases of pulmonary tuberculosis in over 700 CF patients they reviewed. Hoiby (91) reported that 1.3% of patients at a Danish CF center were colonized with atypical mycobacteria. Boxerbaum (23) found that 8 of 430 CF patients were infected with rapidly growing mycobacteria, six with *Mycobacterium chelonei* and two with *M. fortuitum*. *M. chelonei* infection was believed to be the cause of death in one of the six patients. Smith et al. (170) found that 7 of 223 CF patients were infected with mycobacteria: three with *M. tuberculosis*, two with rapid growers (one each *M. fortuitum* and *M. chelonei*), and two with unidentifiable mycobacteria. Two of three patients with *M. tuberculosis* were symptomatic and were treated successfully; the third, who was asymptomatic, was not treated. The patient with *M. fortuitum* died despite chemotherapy and the organism was recovered from lung tissue at autopsy. The patient with *M. chelonei* improved radiographically and clinically after combination therapy with rifampin, isoniazid, amikacin, and erythromycin.

Kinney et al. (102) have recently described a case of *M. avium* complex infection in an adult CF patient who received a heart-lung transplant. The lungs removed during transplantation showed extensive granulomas with "caseous necrosis and liquefaction." The granulomas contained many acid-fast bacilli. We have found *M. avium* complex in the sputum specimens of 12 of 59 adult CF patients. Preliminary studies suggest that, in one of these patients, this agent was associated with a decline in lung function (101a). As life expectancy increases in CF patients, colonization with atypical mycobacteria may become more prominent. In some, these organisms may contribute to lung deterioration.

Culturing mycobacteria from sputa of CF patients can be difficult. CF patients colonized or infected with mycobacteria often are also infected with *P. aeruginosa*. *P. aeruginosa* can survive decontamination procedures used to eliminate other bacterial flora in mycobacterial respiratory tract cultures. Because of this, they can overgrow the mycobacterial cultures as well as degrade and liquefy Lowenstein-Jensen agar, making mycobacterial isolation impossible (170). As a result, the importance of direct microscopic examination of respiratory secretions for acid-fast bacilli must be emphasized. Although mycobacteria appear to play a minor role in the lung disease of CF patients, they should be considered in the differential diagnosis in CF patients with characteristic symptoms of mycobacterial infection.

Unculturable agents. One of the dilemmas in caring for CF patients is trying to determine the cause of pulmonary exacerbation when no obvious changes have occurred in the type and number of organisms present in their respiratory secretions. This is a particular problem in patients who have long-term colonization or infection with mucoid *P. aeruginosa*. One of the hypotheses to explain this finding is that the exacerbation is due to some unculturable agent. The observation that antimicrobial therapy with agents that are essentially inactive against *P. aeruginosa* results in improved clinical status supports this theory (120). *P. aeruginosa* might obscure the growth of those microbial agents against which this antimicrobial therapy might be effective. Examples of slowly growing or difficult to isolate organisms include *H. influenzae* and *P. cepacia*, which can play a role in pulmonary exacerbations in CF patients.

In the early 1980s, data suggested that *L. pneumophila* might be another organism that would be extremely difficult to culture in the presence of *P. aeruginosa* and might be responsible for pulmonary exacerbations in this population. These data, generated by two groups (59, 98), were based on serologic responses to *L. pneumophila* antigens as measured by an indirect fluorescent-antibody test. In one study (59), titers of >16 were found in 8 of 46 CF patients but only one patient had a fourfold rise in titer. In the other study (98), 32 of 109 CF patients had antibody titers of >128. These investigators found that patients with high antibody levels had more severe pulmonary disease than those with low titers. Later work by Collins et al. (41) showed that *P. aeruginosa* shares several common antigens with *L. pneumophila*. Tenover et al. (186) showed that polyclonal antibodies against *L. pneumophila* cross-react with several *Pseudomonas* species, including *P. aeruginosa*. These data suggest that the antibody titers seen in CF patients may represent cross-reactions with *Pseudomonas* sp. or perhaps other organisms that colonize the respiratory tree. Gene probes for *Legionella* sp. are now widely available. Although false-positive results have been reported with this technique (111), use of this test may help determine the role of *Legionella* spp. in CF lung disease.

Anaerobic bacteria might also play a role in pulmonary infection of CF patients. Protected bronchial brush specimens, transtracheal aspirates, and lung tissue are optimal specimens for culture of anaerobes but are seldom obtained. Consequently, little data are available on the role of anaerobes in pulmonary disease of CF patients. Thomassen et al. (193) studied 17 patients who underwent thoracotomy and compared tissue or aspirates obtained from that procedure with sputum cultured quantitatively for both aerobes and anaerobes. Two of 10 patients studied for the presence of anaerobes in both sputum and thoractomy specimens had them in both. In both specimens, *P. aeruginosa* was present at levels similar to those of the anaerobes, suggesting that anaerobes had a secondary role in these infections. Much

more information is required before the role of anaerobes in CF patients can be determined.

Fungal agents. The major fungal agent responsible for pulmonary disease in CF patients is *Aspergillus fumigatus*. Specifically, this organism causes allergic bronchopulmonary aspergillosis (ABA). Invasive aspergillosis and aspergilloma are unusual in this population (30, 165, 196). The prevalence of ABA is estimated to be between 0.5 and 11% in CF patients and is believed to occur primarily in older children and young adults (30, 121). Generally, disease in CF patients is sporadic, but a cluster of cases has been described (121).

The diagnosis of ABA is primarily clinical with the characteristic findings of bronchoconstriction, pulmonary infiltrates, eosinophilia, increased serum IgE levels, skin test reactivity, and serum precipitins to *A. fumigatus* (196). The recovery of aspergillus from respiratory secretions supports the diagnosis, but it has been estimated that as many as 60 to 80% of CF patients can be colonized with this fungus (196). Antibodies to *A. fumigatus* are also common. Surveys of *Aspergillus* precipitins (154, 165, 166) have shown that these antibodies are present in 31 to 37% of CF patients, suggesting that a positive serologic test supports but does not confirm diagnosis of ABA. Corticosteroids are the treatment of choice for ABA (30, 196).

Candida albicans frequently can be recovered from the respiratory tree of CF patients (154), especially those receiving antimicrobial agents and steroids. However, the role, if any, that this organism plays in lung disease of these patients has not been delineated.

Viruses. The role of viruses in the evolution of lung disease in CF patients has not been determined. It has been postulated that viral respiratory tract infections are important in predisposing the CF lung to bacterial infection (143, 157). It is known that ciliary function is normal, at least early in life, in patients with CF (199). Carson et al. (32) have shown that viral respiratory tract infections can disrupt ciliary function. Disruption of ciliary function may then lead to the establishment of secondary bacterial infections and, by a process yet to be discerned, chronic infection in CF patients. As is seen in non-CF children (47), viral lower respiratory tract infections caused by influenza and parainfluenza viruses and respiratory syncytial virus (RSV) are common in patients with CF (1, 143, 200). Unfortunately, much of the data that support this observation are based on serologic rather than viral culture results.

Wang et al. (200), for example, studied 49 patients with CF over a 2-year period. During that study, they performed viral cultures on 1,046 nasal wash specimens, all of which were negative. Nevertheless, 105 viral infections were detected by serology, with 40% being asymptomatic. They also found that CF patients with >1.67 viral infections per year had a more rapid progression in lung disease than those with fewer viral infections. Petersen et al. (143), using serologic data only, suggested that viral infections, especially RSV, were more common in patients who developed chronic *P. aeruginosa* infection.

The best study to date on the role of viruses in lung disease in CF patients is by Abman and colleagues (1). Forty-eight children were studied longitudinally from the time the diagnosis of CF was established. Eighteen children were hospitalized 30 times for acute respiratory distress. Twelve children had a viral pathogen detected, seven of whom had RSV. Of the seven RSV infections, four were detected by culture. Of these four, two were also detected by a direct fluorescent-antibody test for RSV antigens. The remaining three were diagnosed serologically. The seven patients with RSV infection had more signs of chronic respiratory disease and lower radiographic scores than the other CF infants. These data indicate that viral infections, especially those caused by RSV, may be responsible, in part, for deteriorating lung function in young children with CF. Two other points are relevant in regard to the study by Abman et al. One is that direct fluorescent-antibody test for RSV, which has only recently become available, may be of great value in diagnosing viral respiratory tract infections in CF patients. Nucleic acid hybridization techniques may also prove useful for detecting viruses in situ, especially in children colonized with *P. aeruginosa*, which readily contaminates cell cultures used for virus isolation. Second, Abman et al. emphasize the potential value of antiviral agents in preventing lung damage due to viral infection which may have long-term consequences in this patient population.

STRATEGIES FOR CULTURE OF RESPIRATORY SECRETIONS FROM CF PATIENTS

Specimen Collection

The range of microbes that infect the airway of CF patients presents a challenge for the clinical microbiologist. In young children with CF, sputum is not produced. As a result, alternative strategies are used for obtaining material for culture in this age group. The most commonly used technique in the young child with CF has been the throat swab or "gagged" sputum. In this method, a swab is placed in the pharynx to gag the child. A paroxysm of coughing may then ensue, resulting in lung secretions being coughed onto the swab. The flora cultured by this technique may represent only normal throat flora, but the throat flora in these young children may reflect the flora in the lung airways. A longitudinal prospective study of lung infection in infants and young children with CF is being performed at our institution to begin addressing this issue. In this study, we are comparing the flora found on throat swabs taken before bronchoscopically obtained cultures to determine whether upper airway colonization corresponds to the organisms found in the lower airways. This study should determine the diagnostic value of gagged sputum specimens.

In older children and adults, sputum production, especially during pulmonary exacerbations, is usually copious. As a result, obtaining sputum that meets criteria for a "good specimen," i.e., a >1 ratio of white blood cells/epithelial cells on Gram stain (17, 129), is easily accomplished. Further, studies by Thomassen and colleagues (193) have shown that sputum cultures obtained from CF patients predict the microflora found in the lung as evidenced by comparing sputum cultures with culture of lung tissues or aspirates obtained during thoracotomy. In their study, 15 of 17 patients had the same organism in sputum and lung tissue or aspirates. The other two had a positive sputum but negative aspirate. These data suggest that sputum cultures in older CF patients generally reflect the flora of the lower airways.

Quantitative Sputum Cultures

Two reasons have been advanced for the use of quantitative sputum cultures. First, it is thought that quantitation of bacterial numbers is an appropriate means to measure the efficacy of antimicrobial therapy. This technique, therefore, has been used in a number of studies (10, 19, 76, 107, 120, 127, 169, 209), especially to measure efficacy of antipseu-

TABLE 2. Suggested media and incubation conditions for routine culture of respiratory secretions from patients with CF

Medium	Incubation conditions	Organisms detected
Chocolate/horse blood agar with bacitracin or	35–37°C for 3–4 days, ambient air	*H. influenzae*
Horse blood agar	35–37°C for 2–3 days, anaerobically	*H. influenzae*, *Streptococcus pneumoniae*, *S. pyogenes*
Mannitol-salt agar	35–37°C for 2–3 days, ambient air	*Staphylococcus aureus*
MacConkey agar	35–37°C for 2–3 days, ambient air	*P. aeruginosa*, other *Pseudomonas* spp.; *Enterobacteriaceae*
PC or OFPBL agar	35–37°C for 3–4 days, ambient air	*P. cepacia*
Sabouraud agar with antibiotics	30 or 35°C for 3–7 days, ambient air	Yeasts, molds

domonal therapy. Decline in pseudomonal numbers generally, but not always, parallelled improved clinical outcomes. One glaring exception was found in patients with *P. cepacia*. Numbers of *P. cepacia* rarely declined in the face of intensive antipseudomonal therapy despite improvement in clinical condition. In some patients, clinical improvement was concurrent with decline in *P. aeruginosa*. In others, however, *P. cepacia* was the only potential pathogen present (76).

The other situation in which quantitative cultures appear to be of value is in the isolation of selected organisms. Wong et al. (209) have found that quantitative cultures may be useful in enhancing the recovery of *H. influenzae*, especially in sputum from adult patients. In addition, enhanced recovery of antimicrobial agent-resistant strains of *P. aeruginosa* by using quantitative methods has been reported (119).

Sputum Liquefaction

The technique used for quantitation of bacteria in sputum has varied from study to study. Since CF sputum is highly viscous, the use of a liquefying agent such as *N*-acetylcysteine or dithiothreitol for liquefaction of sputum has been important for obtaining reproducible quantitation. Both agents yielded acceptable results when used with sputum specimens of patients with CF (85, 209). However, in vitro studies by Parry and Neu (136) showed that the concentration of *N*-acetylcysteine used to liquefy sputum inhibited the growth of 9 of 13 *P. aeruginosa* isolates, whereas Hammerschlag and colleagues (85) reported that the concentration of dithiothreitol (50 μg/ml) used to liquefy sputum delayed but did not inhibit the growth of selected strains of *H. influenzae* type b, *P. aeruginosa*, and *S. aureus*. Mechanical dispersion of sputum by an electric homogenizer has also been used for quantitation in clinical studies (77, 80). We recently compared mechanical dispersion versus chemical liquefaction. Microbial recovery was slightly higher with mechanical than chemical liquefaction. All results, however, were within 1 log dilution, indicating that either liquefaction strategy is acceptable as long as it is consistently used (88a).

Strategies for Routine Culturing of Respiratory Secretions from CF Patients

Sputum or gagged throat swabs are usually cultured qualitatively to determine the etiologic agent of acute exacerbations of pulmonary disease in CF patients. The strategy at our institution is to use different media directed at the isolation of specific organisms that are associated with these exacerbations.

For the recovery of *S. aureus*, the use of mannitol-salt agar is strongly advocated. Multiresistant *P. aeruginosa* strains will not obscure the growth of *S. aureus* on this medium as they might on nonselective media or on gram-positive selective media such as colistin-nalidixic acid agar. Also, as previously mentioned, mannitol-salt agar readily supports the growth of thymidine-dependent *S. aureus* and enhances the recovery of this organism.

For the recovery of *H. influenzae*, we use anaerobic isolation on horse blood agar. This technique has several advantages: (i) it suppresses the growth of *P. aeruginosa* which may obscure the growth of *H. influenzae* when isolation is attempted on horse blood agar incubated aerobically; (ii) hemolytic haemophili can be easily distinguished from nonhemolytic species on this medium; (iii) both *S. pneumoniae* and group A streptococci can be isolated with this technique, although both are unusual causes of pulmonary exacerbations in CF patients (91, 92, 96).

For isolation of *P. aeruginosa* and other glucose fermenters and nonfermenters, MacConkey agar is adequate. For isolation of *P. cepacia*, PC agar has been reported to be superior to other commonly available media (74). Alternatively, OFPBL agar can be used for *P. cepacia* isolation.

Aspergillus sp. and *Candida* sp. may also play a role in pulmonary exacerbations and their isolation should be attempted routinely. Sabouraud agar containing chloramphenicol and cycloheximide may be used. However, *P. aeruginosa* may overgrow fungi on this medium. Generally, incubation for 3 days is sufficient to isolate most common pathogens, including yeasts and molds, although isolation of unusual bacteria and fungi may require longer incubation times. Recommended media and incubation times for isolation of respiratory pathogens from CF patients are summarized in Table 2.

The detection of *Legionella* sp., *Mycobacterium* sp., and viruses present a particular challenge to the clinical microbiologist. Attempts to isolate these organisms are often thwarted by the presence of *P. aeruginosa*, which can contaminate viral cultures or overgrow bacterial ones. Because of this, the development of gene probes and fluorescent-antibody reagents for direct detection of these agents is particularly attractive for diagnosis of respiratory infections in CF patients.

CONCLUSION

As the effectiveness of antimicrobial therapy for pulmonary exacerbations in CF patients continues to improve, life expectancy of these patients lengthens. Monitoring the respiratory tract flora and understanding its pathogenic potential will allow the development of new treatment strategies which may further prolong the lives of these patients. Understanding of the basic cellular defects in CF should

allow for the development of novel strategies for prevention of chronic lung infection in patients with CF.

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