Pseudomonas aeruginosa: Immune Status in Patients with Cystic Fibrosis

R. G. DOGGETT AND G. M. HARRISON

*Bacteriology and Immunology Research Laboratory, Texas Institute for Rehabilitation and Research,
Departments of Rehabilitation and Pediatrics, Baylor College of Medicine, Houston, Texas 77025*

Received for publication 5 May 1972

In order to have a better understanding of the clinical significance of *Pseudomonas aeruginosa*, circulating and secretory antibodies were measured. Of 100 patients diagnosed as having cystic fibrosis (CF) and an atypical mucoid *P. aeruginosa* cultured from their sputum, each possessed serum precipitins. These immunoprecipitates, however, were not detected in the sera of 40 CF patients, some of whom were chronically ill with pulmonary colonization by typically rough-smooth strains of *P. aeruginosa*. The sera of 46 CF patients and 27 CF patient parents not colonized by *P. aeruginosa* were negative for the precipitins. The sera from 15 of 45 chronically ill patients not having CF, however, but harboring *P. aeruginosa*, also possessed serum precipitins. The sera from 85 subjects not having CF and not clinically infected with *P. aeruginosa* were negative for precipitins. Serum hemagglutination titers as high as 1:4096 were measured in older CF patients having advanced pulmonary disease and who were infected with mucoid *P. aeruginosa*. Salivary titers ranged from 1:8 to 1:64. Increased levels of both circulating and secretory antibodies of the immunoglobulin A and G classes were demonstrated in patients with CF. Once a patient with CF becomes colonized with *P. aeruginosa* a process of conversion from the rough and smooth forms to the mucoid form is almost inevitable. Although the mucoid form predominates in the sputum, intermediates of the various colony types are often present. Serum precipitins were demonstrable only after the appearance of mucoid strains in the sputum of patients with CF. Although antibiotics tend to reduce the number of mucoid microorganisms, they are rarely, if ever, eradicated from these patients' lungs. Recurrent episodes of severe pulmonary infection and the evidence of increasing antibody formation to mucoid strains indicates the invasiveness of these particular strains.

Recurrent pulmonary infections are recognized as the major threat-to-life complications in most patients with cystic fibrosis (CF). Anderson (1), in 1938, described the morbid pathology seen in the lungs in patients with CF and stated that, in each of the 44 cases studied, there was evidence of respiratory infection. Blackfan and May (2) that same year described 35 cases of CF of the pancreas in which all patients died at an average age of 8 months and with widespread pulmonary infection. *Staphylococcus aureus* was the major pathogen responsible for these early infections. Longevity has increased with earlier detection and better pulmonary cleansing therapy; however, the major contribution to the prolongation of life has been the advent of antibiotics. Although morbidity has improved over the past decades, the general course is still one of steady deterioration. This is due, primarily, to pulmonary infections resulting from a combination of both antibiotic-resistant microorganisms and an ap-

parent intrinsic defect in bacterial-clearance mechanisms.

Although respiratory infections are paramount in CF, evidence of septicemia is uncommon. Positive blood cultures are seldom obtained prior to death, even in the presence of superinfection resulting in massive pulmonary damage. The ability of these patients to provide adequate systemic protection, while their respiratory tract is highly vulnerable to infection, poses a situation which questions certain aspects of their respiratory defense mechanisms, namely, the secretory immune system. An investigation was conducted to determine specific circulatory and secretory antibodies and to compare the relative concentrations of such antibodies in the following major immunoglobulin (Ig) classes: IgG, IgM, and IgA.

Pseudomonas aeruginosa, particularly atypical mucoid strains, is now emerging as the predomi-

nant pathogen associated with the terminal pulmonary manifestations in patients with CF. (8). Antigens prepared from different strains were used to detect specific circulating and secretory antibodies and to measure certain parameters of the immune response in the presence of progressive lung disease.

MATERIALS AND METHODS

Serum samples from 186 patients, from 1 to 30 years of age and with a confirmed diagnosis of CF, and 27 heterozygous parents of CF patients were obtained and tested. Control subjects used were patients hospitalized at the Texas Institute for Rehabilitation and Research, Houston, Texas, and patients treated at the Pediatric Chest Clinic, Texas Children's Hospital, Houston, Texas. These control subjects were being treated for various chronic illnesses, such as other respiratory infections, and some of the subjects were postsurgery patients and urology patients. Whole saliva was collected and maintained at 4 C. The saliva was concentrated from 100 ml down to 5 ml by ultrafiltration, and molecules larger than 50,000 molecular weight were retained with Diaflo membranes (Amicon Corp., Lexington, Mass.). The saliva concentrate was kept at -20 C until tested.

Strains of *P. aeruginosa* were cultured and identified as previously described (13). Strains (no. 260, 254, 276, 226, and 222, representative of serotype groups 1, 2, 4, 6, and 7, respectively, as described by Fisher et al. [15]) were selected for an antigen preparation to detect serum precipitins. Serum precipitins were not detectable in a significant number of patients who were colonized with mucoid *Pseudomonas* when tested with a polyvalent antigen prepared by mixing the strains previously mentioned and crushing them. The need for an antigen preparation possessing additional serotype determinants became apparent in the early phases of this study.

Precipitins were detected in these patients when their own mucoid *P. aeruginosa* was used as antigen. Therefore, a period of testing the patients' own *Pseudomonas* against their serum demonstrated the marked serological heterogeneity among the *Pseudomonas* isolated from both patients with CF and those subjects not having CF. Patients infected with a mucoid strain of *Pseudomonas*, whose sera reacted with their own *Pseudomonas* but failed to react with the antigen pool, had their strain serotyped. Six strains isolated from these subjects, no. 3555-1, 3523, 3482, 3579, 4232, and 3515 which did not cross-react when tested against approximately 100 individual cultures of *Pseudomonas* which were classified as untypable by the Fisher method, were combined with the above typable strains to form a *serotype pool* preparation representing a polyvalent immunotype antigen.

Each of the above strains was used to seed an individual Trypticase soy agar plate. The cultures were incubated for 24 hr at 37 C. Physiological saline (5.0 ml) was added to each plate surface, and the cells were gently scraped from the agar with a glass rod. The bacterial suspension from each of the 11 strains was pooled and three different antigen preparations were

prepared by sonic treatment, crushing the cells with etched glass, and autoclaving at 121 C under 15-psi pressure for 1.5 hr. The suspensions were centrifuged at 10,000 rev/min for 15 min, and the supernatant fractions were lyophilized.

A sample of fresh serum from individuals used in this study was first tested for immunoprecipitating antibodies by using the Ouchterlony double-diffusion technique. Sera exhibiting one or more reactive bands were also tested by using immunoelectrophoresis. An agar buffer, pH 8.2, with an ionicity of 0.075, was prepared by using 47.4 g of sodium barbitol, 690 ml of 0.1 N HCl, and 0.2 g of sodium azide. This was brought to a final volume of 4 liters with distilled water. The electrolyte buffer, pH 7.5, ionicity 0.150, was prepared with 34.4 g of sodium acetate, 34.4 g of sodium barbitol, and 3.88 ml of 12 N HCl. This buffer was brought to a final volume of 4 liters with distilled water. These buffers were prepared and refrigerated for at least 24 hr prior to use. The antigen preparations containing a dry weight of 50 mg of saline per ml were run at 40 ma and 100 v for 1 hr.

Hemagglutination test. Human type-0 erythrocytes (RBC) were used for this study because sheep RBC appeared to hemolyze more readily when sensitized with the *P. aeruginosa* antigens. Purified *P. aeruginosa* capsular polysaccharide (11) was dissolved in hemagglutination buffer (Difco) until saturated. This was used to prepare a 50% (v/v) working solution in the buffer. A 2% suspension of type-0 human RBC in the capsular-polysaccharide working solution was incubated for a period of 2 hr at 25 C and was gently mixed several times during this process. The solution was refrigerated overnight. The sensitized RBC were washed three times in hemagglutination buffer and resuspended, thus making a 2% RBC working solution in the buffer. The autoclaved serotype pool antigen preparation was used to sensitize RBC in a similar manner.

Indirect fluorescent-antibody test (IFA). The IFA procedure was essentially the same as that used by Tourville (22) in the study of naturally occurring antibodies to *Escherichia coli* in human fluids. Fluorescein (Hyland Laboratories, Los Angeles, Calif.) isothiocyanate-conjugated and rhodamine (Cappel Laboratories, Downingtown, Pa.) isothiocyanate-conjugated monospecific goat antiserum to human immunoglobulins were used to detect specific antibodies in serum and saliva. Serotype pool antigen and washed, whole *Pseudomonas* cells were used for the detection of serum and salivary antibodies.

Quantitation of serum and salivary immunoglobulins. Serum and saliva immunoglobulin levels were determined by the antibody-agar technique of Fahey and McKelvey (14). Immunoplates and specific immunoglobulin standards (Hyland Laboratories) were used to assay for IgG, IgM, and IgA. Secretory, 11S IgA standard was used to quantitate the salivary IgA.

RESULTS

Immunoelectrophoresis. The sera of 100 CF patients, each having evidence of pulmonary colonization by mucoid strains of *P. aeruginosa*,

were shown to contain numerous antibodies when subjected to immunoelectrophoresis (Fig. 1). The sera of 86 patients with CF, together with those of 27 heterozygous parents of CF patients not harboring a mucoid strain of *P. aeruginosa*, were negative for precipitins (Table 1). Among these control subjects, five had mucoid strains and serum precipitins, and 45 had nonmucoid strains only, with 10 having serum precipitins.

Figure 1 shows that autoclaving the antigen preparation destroyed a number of the antigens. These heat-labile antigens make up the bulk of the extracellular products; however, it is not known which of these substances are toxic and which are nontoxic at present. By substituting the antigen in the well with whole sera, one can observe that the major serum immunoglobulin classes, G, M, and A, contain antibodies.

Hemagglutination. The hemagglutination test using the serotype pool antigen demonstrated that serum titers of patients with CF ranged from 1:8 to 1:4096, whereas whole saliva never exceeded a titer of 1:16. The sonicated or crushed antigen preparations could not be used for they hemolyzed the RBC. Whether the capsular polysaccharide or the autoclaved serotype pool was used to sensitize the RBC, the titers of the individuals were essentially the same.

Quantitative immunoglobulins. There were no significant differences detected among the CF patients, heterozygous parents, or the controls

in the concentrations of the salivary immunoglobulins. IgM could not be detected in any of the salivary samples. IgA was present in each sample, whereas IgG was found in approximately 50% of the secretions. Each of the three immunoglobulin classes was found to be increased in the serum of patients with CF. IgG and IgA were consistently increased in comparison with the controls, whereas IgM was not significantly increased.

Fluorescent-antibody test. Anti-*Pseudomonas* antibodies were detected among the IgG, IgM, and IgA serum immunoglobulins. Normal, newborn cord sera and control sera were negative by the indirect fluorescent-antibody test. The same technique applied to the salivary samples showed that IgA gave the most brilliant fluorescence when treated with fluorescein-conjugated or rhodamine-conjugated antiserum to IgA. Various degrees of fluorescence (0 to 2+) were observed in the IgG and IgM fractions. A much stronger fluorescence was observed in the washed, whole *P. aeruginosa* cells than in the heat-treated serotype pool preparation in the IgA salivary fraction.

DISCUSSION

Sera from patients with CF has been investigated for *P. aeruginosa* antibodies by Doggett et al. (8, 10), Schwartz and Virtanen (19), Burns and May (3) and, more recently, by Diaz, Mosovich, and Neter (6). It is generally agreed that

FIG. 1. Comparative immunoelectrophoresis of the three antigen preparations that were placed in the wells and subjected to electrophoresis. The serum from one patient with CF was placed in the troughs. The antigens were A, sonicated; B, autoclaved; and C, crushed cells. Placing the patient's serum in the well, subjecting the serum to electrophoresis, and putting the antigens into the trough demonstrates that the antibodies are more concentrated in IgG (D, E, F).

TABLE 1. Occurrence of *Pseudomonas*-specific serum precipitins in different subject groups^a

Subject group	No.	Mucoid strain with		Nonmucoid strain only with	
		Orga-nisms (no.)	Serum precipitins (no.)	Orga-nisms (no.)	Serum precipitins (no.)
CF	100	100	100	0	0
CF	86	0	0	40	0
Parents of CF subjects	27	0	0	0	0
Control (chronically ill non-CF subjects)	130	5	5	45	10

^a Microorganisms were in sputum. CF, Cystic fibrosis.

serum antibodies to *Pseudomonas* antigens are more common in those CF patients infected by mucoid strains of *P. aeruginosa*. The report of Burns and May (3) suggests that CF patients with mucoid strains of *P. aeruginosa* in their sputum possess *Pseudomonas*-specific precipitins and that the detection of such serum precipitins can be used as evidence of pathogenicity in such patients. This supports our earlier contention (8, 12) that atypical mucoid forms of *P. aeruginosa* were significant in the maintenance of the patients' pulmonary disease. These present findings have shown that patients with CF, without serum precipitins or mucoid *Pseudomonas* in the sputum, generally reflected a better clinical condition than did those having CF and possessing serum precipitins and mucoid *Pseudomonas*. The patients with CF who had high hemagglutination titers both to serotype pool and capsular polysaccharide antigens had clinical evidence of severe pulmonary disease. It has also been noted in this investigation that an increasing serum titer to *Pseudomonas* reflects a poor prognosis. Eleven of the patients with the higher titer have since expired due to respiratory insufficiency, with mucoid *Pseudomonas* being the final pathogen cultured not only from their sputum but also throughout their lungs shortly after death. Necropsy revealed that the lungs were filled with tenacious mucus and that many airways were plugged. Necrotic abscesses filled with purulent material were numerous throughout the lungs. Pure cultures of mucoid *P. aeruginosa* were cultured from these sites. Clinical and laboratory findings on these subjects prior to death failed to

show evidence of sepsis in spite of the massive pulmonary infection. It is not presently known whether chemotherapeutic antibiotics are responsible for this or if antibodies to *Pseudomonas* provide systemic protection. Apparently, little defense to the pulmonary tree against mucoid *Pseudomonas* is provided by either.

Hypergammaglobulinemia has been well documented in patients with CF and has been demonstrated by Schwartz (20) to be due to the elevation of the IgG and IgA serum immunoglobulins. South et al. (21) reported that the mean serum IgA levels were elevated in children with CF in contrast to normal controls. Martinez-Tello et al. (17) described a significant increase in the number of bronchial mucosa cells containing IgA and IgG in patients with CF and attributed this to severe bronchial inflammation.

The present findings suggest that the immunoglobulins present contain antibodies to *P. aeruginosa*. By using immunoelectrophoresis and fluorescent-antibody techniques, the serum immunoglobulins, IgG, IgM, and IgA, were found to contain specific antibodies. Although the relative concentrations of anti-*Pseudomonas* antibodies were greater in the serum IgG and IgM classes, secretory IgA was prominent in saliva.

Because it was determined that the immunoglobulin concentration is considerably affected by the rate of salivary flow and inflammation increases the transfer of most serum proteins into saliva, the results of the quantitation of the salivary immunoglobulins at this time appeared meaningless.

Of particular interest was the finding that *Pseudomonas*-specific precipitins could not be demonstrated in the sera of 40 patients with CF who harbored rough nonmucoid strains of *P. aeruginosa* in their sputum. This finding has several important implications concerning the role of *P. aeruginosa* in contributing to pathogenesis in this disease. It is generally recognized that microbial immunogens have to pass through the mucous epithelium into the lamina propria in order to evoke a considerable immune response. Accordingly, it appears that rough strains of *P. aeruginosa* represent surface or lumen colonization in this disease, whereas mucoid strains possess properties enabling these microorganisms to invade the respiratory lamina propria, thereby reaching the subject's immune system. Rough strains, no doubt, enter the lamina propria after tissue damage has occurred but are readily phagocytized by macrophages. One could speculate that the mucoid microorganisms are protected during phagocytosis by a thick coat of alginic acid (4, 16; Fig. 2, 3, and 4). This extracellular polysaccharide probably resists rapid enzymatic

FIG. 2. Polymorphonuclear neutrophil from a patient with CF with a serum hemagglutination titer of 1:4096. Peripheral blood was incubated with mucoid *P. aeruginosa* for 30 min. The buffy coat was fixed with 2.5% glutaraldehyde and postfixed with 2% osmium buffered with phosphate. The sections were stained with lead citrate. Magnification, $\times 10,000$.

degradation, because human tissue is not known to possess alginase. Additionally, it has been shown that the capsular polysaccharide of these mucoid strains is toxic to mouse (8) and chicken embryos (5) and that this toxic property is lost when the polysaccharide is degraded by alginase (5).

It is noteworthy to mention that although the lungs are predisposed to infection almost from birth, other areas of the body are not. We have

never isolated *Pseudomonas* from the urine, feces, tears, or sweat of patients with CF. This does not mean that they cannot become infected. The parents and siblings that we have cultured were not colonized by *Pseudomonas* nor could we detect any evidence that they had been in the past.

The patients used in this study who did not have CF, but had *Pseudomonas* infections, are discussed by Doggett et al. (9) elsewhere.

We have observed a number of CF subjects

FIG. 3. Electron micrograph of sample used in Fig. 2 with the incubation time extended to 4 hr before fixation. Note that a few of the microorganisms are still intact and that one bacterium has been completely destroyed in the phagocytic vacuole. Magnification, $\times 10,000$.

referred to this center for diagnostic or therapeutic planning purposes who had no prior history of continuous "prophylactic" antibiotic therapy, or an inhalation therapy program, but who were already colonized by mucoid strains of *Pseudomonas*.

The reason individuals with CF are so vulnerable to pulmonary infections and why *Pseudomonas* emerges into a mucoid form in almost all of these patients remains unknown. By the

number of different antibodies produced, it is apparent that the immune systems of these individuals are responding to different immunogenic substances. Today, with proper chemotherapy these patients' staphylococcal infections can be managed successfully. However, *Pseudomonas* presents a different task. Mucoid strains appear to have several means to confer virulence: first, by direct effects of toxic substances on tissue and, secondly, by an obstructive mechanism

FIG. 4. Polymorphonuclear neutrophil from a normal, healthy subject. Incubation time was 6 hr. Two bacteria (arrows) are in the process of cell division while inside the phagocytic vacuole. Magnification, $\times 9,000$.

whereby the viscid slime produced by these microorganisms obstructs airways in the lungs leading to respiratory insufficiency.

We have noted that some strains appear more virulent than others and that a suitable typing system is needed to determine when these virulent strains emerge so that increased chemotherapy can be initiated. We are now in the process of developing such a system.

ACKNOWLEDGMENTS

We thank Valentina Immer, Stanley Gunsberg, Judy Allen, Susan Vincent, and Janis Gwin for their excellent technical assistance.

This investigation was supported by Research and Training Center project RT-4 from the Social and Rehabilitation Service, the Houston Endowment Foundation, and the National Cystic Fibrosis Research Foundation.

LITERATURE CITED

1. Andersen, D. H. 1938. Cystic fibrosis of the pancreas and its relation to celiac disease: a clinical and pathologic study. *Amer. J. Dis. Child* 56:344-399.
2. Blackfan, K. D., and C. D. May. 1938. Inspissation of secretion, dilatation of the ducts and acini, atrophy and fibrosis of the pancreas in infants. *J. Pediatr.* 13:627-634.
3. Burns, M. W., and J. R. May. 1968. Bacterial precipitins in serum of patients with chronic illness. *Lancet* 1:270-272.
4. Carlson, D. M., and L. W. Matthews. 1966. Polyuronic acids

- produced by *Pseudomonas aeruginosa*. *Biochemistry* 5:2817-2822.
5. Cundy, K. R., and R. J. Mattson. 1969. Toxicity as capsular polysaccharide and endotoxin from mucoid strains of *Pseudomonas* isolated from cystic fibrosis. *Bacterial. Proc.*, p. 88.
 6. Diaz, F., L. L. Mosovich, and E. Neter. 1970. Serogroups of *Pseudomonas aeruginosa* and the immune response by patients with cystic fibrosis. *J. Infect. Dis.* 121:269-274.
 7. Doggett, R. G., 1969. Incidence of mucoid *Pseudomonas aeruginosa* from clinical sources. *Appl. Microbiol.* 18:936-937.
 8. Doggett, R. G., and G. M. Harrison. 1969. Significance of the bacterial flora associated with chronic pulmonary disease in cystic fibrosis, no. 17. In 5th International cystic fibrosis conference. Cambridge.
 9. Doggett, R. G., G. M. Harrison, and R. E. Carter. 1971. Mucoid *Pseudomonas aeruginosa* in patients with chronic illness. *Lancet* 1:236-237.
 10. Doggett, R. G., G. M. Harrison, R. N. Stillwell, and E. S. Wallis. 1965. Antibody response to *Pseudomonas aeruginosa* infections associated with cystic fibrosis of the pancreas. *S. Med. J.* 58:1595.
 11. Doggett, R. G., G. M. Harrison, R. N. Stillwell, and E. S. Wallis. 1965. Enzymatic action of the capsular material produced by *Pseudomonas aeruginosa* of cystic fibrosis origin. *J. Bacteriol.* 89:476-480.
 12. Doggett, R. G., G. M. Harrison, R. N. Stillwell, and E. S. Wallis. 1966. An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. *J. Pediat.* 68: 215-221.
 13. Doggett, R. G., G. M. Harrison, and E. S. Wallis. 1964. Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis. *J. Bacteriol.* 87:427-431.
 14. Fahey, J. L., and E. M. McKelvey. 1965. Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* 94:84-90.
 15. Fisher, M. W., H. B. Devlin, and F. J. Gnabaski. 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. *J. Bacteriol.* 98:835-836.
 16. Linker, A., and R. S. Jones. 1964. A polysaccharide resembling alginic acid from a *Pseudomonas* microorganism. *Nature (London)* 204:187-188.
 17. Martinez-Tello, F., D. G. Braun, and W. A. Blanc. 1968. Immunoglobulin production in bronchial mucosa and bronchial lymph nodes, particularly in cystic fibrosis of the pancreas. *J. Immunol.* 101:989-1003.
 18. Reid, L. 1968. Disturbances in the pattern of secretion of bronchial mucous glands, p. 45-55. In R. Porter and M. O'Connor (ed.), *Cystic fibrosis*, Ciba Foundation study group no. 32. Little, Brown, & Co., Boston.
 19. Schwartz, R. H., and S. Virtanen. 1965. Serologic reactions in patients with cystic fibrosis, p. 9. In Sixth annual cystic fibrosis club report. National Cystic Fibrosis Foundation, Atlanta.
 20. Schwartz, R. J. 1966. Serum immunoglobulin levels in cystic fibrosis. *Amer. J. Dis. Child.* 111:408-411.
 21. South, M. A., W. J. Warwick, F. A. Woolheim, and W. A. Good. 1967. The IgA system III. IgA levels in the serum and saliva of pediatric patients—evidence for a local immunological system. *J. Pediat.* 71:645-653.
 22. Tourville, D., J. Bienenstock, and T. B. Tomasi. 1968. Natural antibodies of human serum, saliva, and urine reactive with *Escherichia coli*. *Exp. Biol. Med.* 128:722-727.