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An Immunohistological Evaluation of *Pseudomonas aeruginosa* Pulmonary Infection in Two Patients with Cystic Fibrosis

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ABSTRACT. *Pseudomonas aeruginosa* is the principal pulmonary pathogen in patients with cystic fibrosis. All attempts to date to prevent or eradicate *P. aeruginosa* infections in these patients have been unsuccessful. Vaccination against *P. aeruginosa* has been proposed as a preventive strategy but it has not been adequately evaluated. The purpose of this study was to determine whether *P. aeruginosa*, present in the lungs of patients with cystic fibrosis, express surface antigens similar to those grown *in vitro*; this issue is of critical importance when choosing bacterial products as vaccine candidates. Lung sections from two patients who died of the pulmonary complications of cystic fibrosis were studied. Bacteria, both in lung sections and isolated from the lung sections and grown *in vitro*, reacted strongly with polyclonal and monoclonal antibodies against *P. aeruginosa* mucoid exopolysaccharide and outer membrane proteins F and H2; this suggested that these antigens are surface exposed *in vivo*. It was also found that bacteria in both lung sections were associated *in situ* with IgG, IgA, and C3 but not with IgM or C4. (*Pediatr Res* 22: 743–747, 1987)

Abbreviations

CF, cystic fibrosis
MEP, mucoid exopolysaccharide
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
LPS, lipopolysaccharide
PBS/FCS, phosphate-buffered saline with 1% fetal calf serum

Pseudomonas aeruginosa is associated with pulmonary infections in most patients with CF (1). Although *P. aeruginosa* is thought to be the principal CF bacterial pathogen, many questions regarding its role in the disease process remain unanswered. The unique host-parasite relationship in the CF lung is characterized by chronic infection that ultimately leads to fatal pulmonary insufficiency. The progressive infection evolves despite the presence of high levels of anti-Pseudomonal immunoglobulins in serum and sputum. Furthermore, CF host defenses appear to be largely intact (2), and CF phagocytic cell function in essentially normal (2). However, serum and sputum anti-Pseudomonal IgG may be deficient in the Fc portion (3). Consequently, it has been suggested that these molecules lack the capacity to opsonize Pseudomonas for ingestion by alveolar macrophages (3).

Observations regarding the pathophysiology of *P. aeruginosa* infections in CF have been derived largely from *in vitro* investigations. The bacteria have been grown on artificial media and might differ significantly from those present *in situ* in the CF lung. Brown *et al.* (4) have demonstrated with *P. aeruginosa* isolated from CF sputum without further subculture that the outer membranes contain, in addition to their normal complement of major proteins, a series of high molecular weight proteins. These proteins are present *in vitro* only after the bacteria are grown on iron-restricted media. However, while these investigations have demonstrated reasonable conservation of outer membrane proteins (with the exception noted above), they do not show that outer membrane protein antigens are exposed *in vivo* and available as targets for immunotherapy.

We were recently presented with the opportunity to evaluate antigens expressed by bacteria present *in situ* in the lungs of two patients with CF. Both patients died as the result of chronic progressive pulmonary disease and had postmortem examinations. We compared their bacteria grown *in vitro* and present *in situ* in lung sections in terms of reaction with antibodies directed against specific *P. aeruginosa* surface components. We also investigated the nature of the immunoglobulin present on the bacteria in these lung sections. The observations from these studies form the basis of this report.

MATERIALS AND METHODS

Case histories. Patient 1. An East Indian girl was diagnosed as having CF in the neonatal period by the serum immunoreactive trypsin test. This was confirmed by a sweat chloride of 104 and a sweat sodium of 74 mEq/liter. Her initial problems were associated with gastroesophageal reflux that failed to respond to medical or surgical therapy. She developed persistent and progressive pulmonary infiltrative disease that was probably exacerbated by aspiration of gastric contents. Culture of her throat first grew *Pseudomonas maltophilia* at 13 months of age. *P. aeruginosa* was first recovered from a throat culture at 36 months of age. Both organisms persisted in her respiratory secretions until the time of death. She received multiple courses of antibi-

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otics including ticarcillin, tobramycin, chloramphenicol, and moxalactam but the *P. maltophilia* became resistant to all drugs. Since *P. aeruginosa* infection in the patient's lungs was documented for only 3 months prior to death, we consider the patient's colonization by *P. aeruginosa* to be atypical of the usual CF lung infections. In agreement with this, mucoid *P. aeruginosa* strains that often appear only after some years of chronic infection, were not recoverable from the lungs of this patient. She died from pulmonary insufficiency at 39 months of age. Her tracheobronchial tree was extensively plugged with mucopurulent material that grew *P. maltophilia* and two nonmucoid strains of *P. aeruginosa* differentiated on the basis of colonial morphology.

Patient 2. A Caucasian girl was diagnosed as having CF at 16 months of age when she presented with a history of recurrent respiratory infections and malabsorption. Her sweat chloride was 119 and sweat sodium was 99 mEq/liter. She was treated with pancreatic enzyme replacement, physiotherapy, and intermittent anti-Staphylococcal antibiotics. *P. aeruginosa* was first recovered from her sputum at 7 yr of age but was not persistently present until she was 10 yr old. Mucoid *P. aeruginosa* was recovered from sputum when she was 11 yr of age and persisted until the time of her death. She was first admitted to the hospital for treatment of a pulmonary infection at 10 yr of age and readmitted 12 times over the next 5 yr for therapy with intravenous antibiotics. Her *P. aeruginosa* isolate developed resistance to ticarcillin and tobramycin. On her last hospitalization, she was treated with ciprofloxacin to which her isolate was susceptible. She died from pulmonary insufficiency at 15 yr of age. At postmortem examination, her tracheobronchial tree was plugged with tenacious mucopurulent material that grew mucoid and nonmucoid colonial types of *P. aeruginosa*.

Bacterial strains. Mucopurulent secretions from the distal mainstem bronchus were obtained from patients 1 and 2 at postmortem examination and cultured in the Diagnostic Microbiology Laboratory of B.C.'s Children's Hospital. *P. aeruginosa* was identified by standard means. Mucoid and nonmucoid variants were distinguished by conventional macroscopic criteria (5). The culture from patient 1 yielded two different nonmucoid *P. aeruginosa* colonial variants (C1271C and C1272C) and one strain of *P. maltophilia* (C1273C). The culture from patient 2 yielded a mucoid (C1295M) and a nonmucoid (C1280NM) strain of *P. aeruginosa*. For outer membrane experiments, colonies from primary cultures, obtained as above, were recultured on proteose peptone no. 2 agar, and overnight cultures in proteose peptone no. 2 broth were inoculated from a single colony.

Antibodies to outer membrane proteins. Rabbit antiouter membrane antibodies were produced exactly as described (6). Monoclonal antibodies MA5-8 (antiprotein F) and MA1-6 (antiprotein H2) were prepared against *P. aeruginosa* outer membrane proteins as described (7, 8). These antibodies were isolated by growth of their respective producing hybridomas in ascites in mice (7) and partially purified by ammonium sulfate precipitation.

Antibodies to mucoid exopolysaccharide. Polyclonal rabbit antibodies raised to purified MEP from *P. aeruginosa* strain 2192 were prepared as described previously (9). Affinity purified rabbit antibodies to MEP were prepared from the above sera as described previously (10). Monoclonal mouse antibodies to MEP were derived from BALB/c mice immunized intraperitoneally with killed bacterial cells or MEP (10 μg/injection) on alternating days for 2 wk. Three days prior to the cell fusion, the mice were given a final booster injection of purified MEP. Spleens from immunized mice were fused with myeloma cell line SP2/0-Ag14, an immunoglobulin nonsecreting strain developed for somatic cell hybridization with immune spleen cells. Cultures were screened for growth starting 7 days after fusion. Wells showing growth were screened for antibodies specific to MEP using a previously described ELISA (11). Cells in wells which were identified as secreting MEP-specific antibody were then cloned using a limiting dilution cloning technique. Clones that grew up were screened for antibody production and wells that were positive in the ELISA were expanded to obtain culture supernatants containing monoclonal antibodies specific to MEP. Two such clones (11/5 and 5/5) were used for the studies described herein. The specificity of these monoclonal antibodies for MEP was determined as described previously in an ELISA assay (11) and opsonophagocytic assay (10).

P. aeruginosa outer membrane proteins. Outer membranes were isolated by the one step procedure as described (12). The outer membrane proteins were separated by SDS-PAGE using a 14% acrylamide running gel containing 0.07 M NaCl (12).

Western electrophoretic blots. Outer membrane proteins were transferred from SDS gels to nitrocellulose paper. Immunoblotting was performed as described (8) using monoclonal antibodies specific for *P. aeruginosa* outer membrane proteins F (MA5-8) and H2 (MA1-6).

P. aeruginosa LPS phenotypes. The LPS phenotype of the *P. aeruginosa* strains was examined by SDS-PAGE of whole cell lysates, followed by staining with silver periodate, according to the method of Hitchcock and Brown (13).

P. aeruginosa serotypes. The *P. aeruginosa* strains were serotyped by slide agglutination using commercially prepared antisera (Difco Laboratories, Detroit, MI).

Serum sensitivity testing. The *P. aeruginosa* strains were tested for their sensitivity to serum using the two methods described previously (14).

Immunofluorescence of in vitro grown bacteria. Indirect immunofluorescence was performed as described previously (8). Bacteria were grown overnight on Mueller Hinton agar, harvested with sterile swabs, spread on glass slides, and heat fixed. Working dilutions of rabbit polyclonal antibodies (1:1000) and mouse monoclonal antibodies (1:100) were prepared in PBS/FCS. Bacterial smears were incubated with the antibodies or PBS control at room temperature for 30 min, washed three times with PBS/FCS, and then incubated with a 1:20 dilution of fluoresceinated antirabbit or antimouse F(ab')2-specific IgG (Cappel Laboratories, West Chester, PA) for 30 min at room temperature. The slides were washed four times with PBS/FCS, air dried, and examined at 1000-fold magnification with a Zeiss epifluorescence microscope. The observer was blinded as to the identity of each slide and they were scored as 0 to 4+ as determined by the intensity of the fluorescence.

Immunofluorescence studies of lung tissue. Biopsies of the lung were obtained at autopsy, 3.5 and 8 h postmortem. The lung tissue was immediately frozen in isopentane and chilled in liquid nitrogen. Cryostat sections were prepared for direct immunofluorescence using fluoresceinated antihuman IgG, (Fab specific and Fc specific) and fluoresceinated antihuman IgA, IgM, C3, and C4 (Cappel Laboratories). Control lung tissue was obtained from a 13-yr-old girl with juvenile rheumatoid arthritis and interstitial lung disease. Histological evaluation revealed acute and chronic inflammation of unknown etiology. Sections were examined with a Nikon episcopic fluorescence microscope with blue filter and the intensity of fluorescence was recorded and graded 0 through 4+.

For indirect immunofluorescence, cryostat sections from the lung biopsies were incubated with rabbit polyclonal or mouse monoclonal antibodies. Goat antirabbit IgG and goat antimouse IgG (Cappel Laboratories) were used as the fluoresceinated second antibodies. Specimens were examined and results recorded in the same manner used for the direct immunofluorescence material.

RESULTS

Outer membrane analysis. The outer membrane protein profiles of the four *P. aeruginosa* strains, subjected to three passages *in vitro* after isolation from the lungs of the two patients, were nearly identical as assessed by SDS-PAGE analysis (Fig. 1, *lanes A–D)*, but different from that of the single *P. maltophilia* strain

from patient 1 (*lane E*). Immunoblotting analysis after Western transfer of the SDS-PAGE-separated proteins demonstrated that all four *P. aeruginosa* strains had proteins that reacted with monoclonal antibodies directed against proteins F and H2 (Fig. 1). Similar protein antigens were not demonstrable on the *P. maltophilia* isolate.

The *P. aeruginosa* isolates were assessed with regard to their LPS phenotype. Strains C1271C and C1272C were both nontypable (using the Difco serotyping scheme). C1280NM and C1295M were both polyagglutinable. The four strains were found to be serum sensitive by the two methods utilized. The two nontypable strains had a lower ratio of smooth to rough LPS than our standard (14) laboratory strain of *P. aeruginosa*, H103, when run on SDS-PAGE and stained with silver periodate. The polyagglutinable isolates had approximately the same ratio of smooth to rough LPS when compared with strain H103.

Immunofluorescence of in vitro grown bacteria. All four *P. aeruginosa* isolates reacted with the antibodies directed against whole outer membranes, protein F, protein H2, and the MEP (Table 1). Both mucoid and nonmucoid isolates reacted equally well with the anti-MEP immunoglobulins. The *P. maltophilia* isolate did not react with any of the antibodies except for an equivocal reaction with the anti-MEP Mab 5/5.

Indirect immunofluorescence of lung tissue. Lung tissue sections from both patients with CF reacted with all anti-Pseudomonas immunoglobulins and monoclonal antibodies tested (Table 2). The fluorescence pattern was either in the form of short rods or filaments that were approximately the size of bacteria. The non-CF control failed to fluoresce with any of the antisera tested. The MEP antisera (both monoclonal and polyclonal) reacted with bacteria in both lung specimens despite the fact that mucoid *P. aeruginosa* had not been recovered from the respiratory tract of patient 1.

Direct immunofluorescence of lung tissue. Lung tissue from both patients with CF was incubated with fluoresceinated antihuman immunoglobulins and antihuman C3 and C4 (Table 3). Fluorescence was, as above, in the form of short rods but also frequently as long filaments, a pattern similar to that seen for the anti-Pseudomonas antibodies reported in Table 2. These data indicate, but do not prove, that these human components were associated with the surface of bacteria in the CF lung. Lung tissue from both CF patients fluoresced with antisera to IgG [both F (ab')2 and Fc specific], IgA, and C3. There was minimal fluorescence with antiserum to IgM and none with fluoresceinated anti-C4. The non-CF control lung tissue failed to fluoresce with any of the antisera.

DISCUSSION

The host-parasite relationship characteristic of pulmonary infections in patients with CF is unique (1, 2, 15). Mucoid strains of *P. aeruginosa*, which are frequently extremely susceptible to the bactericidal effect of human serum (14, 16, 17), establish a chronic bronchitic infection. Once colonization is established, these bacteria are virtually impossible to eradicate (1). The chronic infectious process evolves in the presence of an exuberant humoral immune response (2, 18, 19) and an intact phagocytic host defense system (2). All attempts at medical intervention to date have failed to prevent or eradicate these indolent infections.

The control of MEP production by CF *P. aeruginosa* isolates is understood poorly. It is not known why these strains are virtually unique to CF patients and what the trigger is for production of MEP in the CF lung. We have shown previously, and confirm herein, that MEP is present on nonmucoid as well as mucoid colonial variants (20); it is probably simply elaborated in greater quantity by the latter. Both monoclonal and polyclonal antibodies against MEP reacted *in vitro* with nonmucoid as well as mucoid clinical isolates. Furthermore, these antibodies produced immunofluorescence in lung sections from patient 1 who was colonized only with nonmucoid strains.

Immunization with Pseudomonas vaccines is one therapeutic strategy which has been proposed. This approach will probably

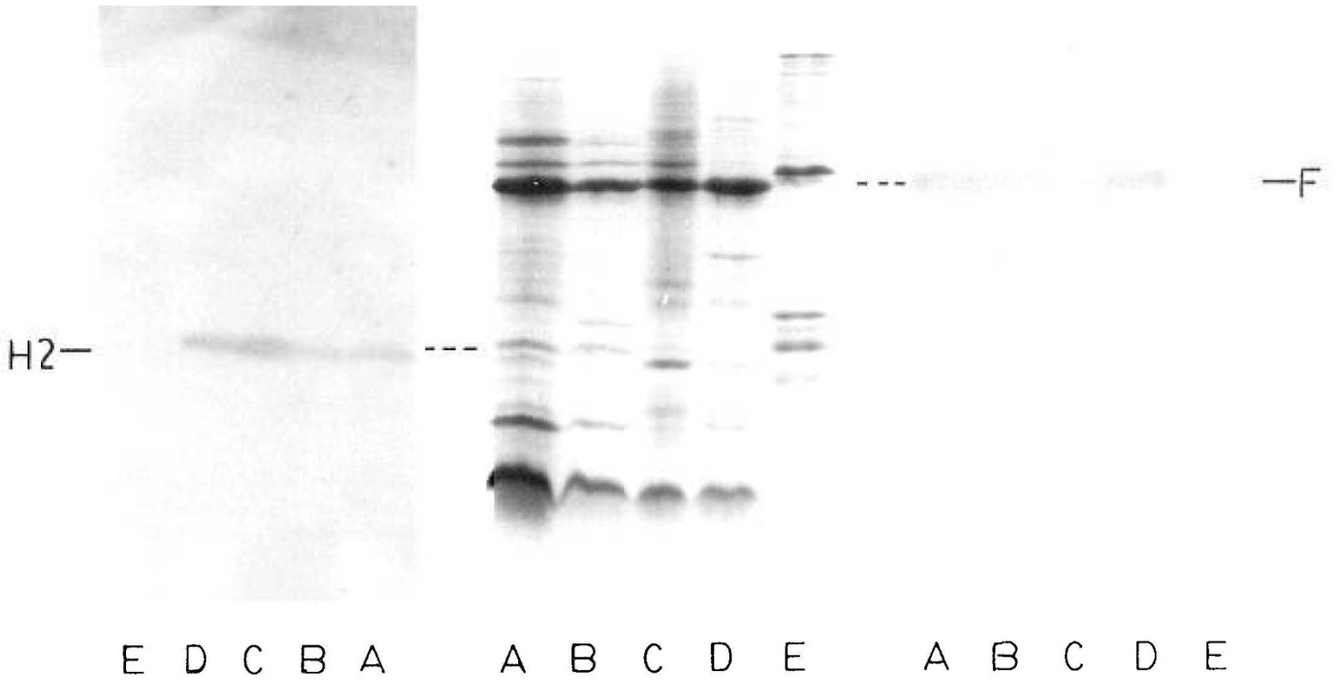

Fig. 1. SDS-PAGE (*middle*), and Western immunoblots following electrophoretic transfer to nitrocellulose (*left and right*), of *P. aeruginosa* C1271C (*lane A*), C1272C (*lane B*), C1280NM (*lane C*), C1295M (*lane D*), and *P. maltophilia* isolate C1273C (*lane E*). The Western immunoblot on the *left* was treated with monoclonal antibody MA1-6, specific for *P. aeruginosa* lipoprotein H2 (6), and the blot on the *right* was treated with monoclonal antibody MA5-8, specific for *P. aeruginosa* outer membrane protein F (7). Small differences in loading accounted for the differences in reactivity on the Western immunoblots.

Table 1. *Indirect immunofluorescence of Pseudomonas isolates from patients with CF*

First antibody	Fluoresceinated second antibody	Patient 1 Bacterial strain *P. aeruginosa* C1271C	*P. aeruginosa* C1272C	*P. maltophilia* C1273C	Patient 2 Bacterial strain *P. aeruginosa* C1280NM	*P. aeruginosa* C1295M
Rabbit antiouter membrane	Goat antirabbit IgG	3+	3+	0	3+	2+
Rabbit anti-MEP	Goat antirabbit IgG	2+	2+	0	2+	2+
Affinity-purified rabbit anti-MEP	Goat antirabbit IgG	2+	2+	0	1+	2+
PBS	Goat antirabbit IgG	0	0	0	0	0
Mouse antiprotein F	Goat antimouse IgG	4+	4+	0	3+	2+
Mouse antiprotein H2	Goat antimouse IgG	2+	3+	0	3+	2+
Mouse anti-MEP (11/5)	Goat antimouse IgG	2+	2+	0	2+	3+
Mouse anti-MEP (5/5)	Goat antimouse IgG	2+	1+	1+/0	2+	2+
PBS	Goat antimouse IgG	0	0	0	0	0

Table 2. *Indirect immunofluorescence of lung tissue from two patients with CF and from one patient without CF*

First antibody	Fluoresceinated second antibody	Source of lung tissue CF patient 1	CF patient 2	Non-CF control
Rabbit antiouter membrane	Goat antirabbit IgG	3+	3+	0
Rabbit anti-MEP	Goat antirabbit IgG	2+	3+	0
Rabbit affinity-purified anti-MEP	Goat antirabbit IgG	4+	1+	0
Mouse antiprotein H2	Goat antimouse IgG	2+	3+	0
Mouse antiprotein F	Goat antimouse IgG	1+/2+	3+	0
Mouse anti-MEP (11/5)	Goat antimouse IgG	0/1+	1+	0
Mouse anti-MEP (5/5)	Goat antimouse IgG	2+/3+	2+/3+	0

Table 3. *Direct immunofluorescence of lung tissue from two patients with CF and from one patient without CF*

Antibody	Source of lung tissue CF patient 1	CF patient 2	Non-CF control
Fluoresceinated antihuman IgG, F (ab')^2 specific	3+	3+	0
Fluoresceinated antihuman IgG, Fc specific	3+	3+	0
Fluoresceinated antihuman IgA	3+	3+	0
Fluoresceinated antihuman IgM	1+	1+	0
Fluoresceinated antihuman C3	4+	2+	0
Fluoresceinated antihuman C4	0	0	0

be of little value after pulmonary infections are already established, by which time patients have mounted a vigorous antibody response to most *P. aeruginosa* products (21). Indeed Hann and Holscaw (22) previously demonstrated that Pseudomonas strains from the sputum of CF patients were coated with IgA, IgG, IgM, and C3. Our results using direct immunofluorescence examination of lung sections agreed in part with these data by showing that the bacteria in lung sections from both CF patients were associated with IgG, IgA, and C3 (Table 3). However, despite the presence of such antibodies in established infections, it can be argued that if opsonizing and/or bactericidal antibody could be elicited prior to pulmonary infection, the devastating pulmonary complications of CF might be prevented or at least forestalled. For immunization to be successful, elicited antibody must recognize surface-exposed bacterial epitopes. The purpose of the studies reported herein was to demonstrate whether or not bacteria present in the CF lung have surface-exposed antigens that are recognized by antibodies raised *in vitro* against purified *P. aeruginosa* products.

We have demonstrated in these studies, that in the cases studied, *P. aeruginosa* strains in the CF lung have at least some antigenic similarities to those grown *in vitro*. Monoclonal and polyclonal antibodies raised against purified *P. aeruginosa* MEP and outer membrane proteins F and H2 bound to the surfaces of bacteria present in lung tissue from patients with CF. These data are consistent with observations that antibodies to these bacterial products are present in the sera of patients colonized with *P. aeruginosa* (9, 23, 24). Thus, there is some potential for

using immunotherapy with these antigens to prevent pulmonary colonization with *P. aeruginosa* of CF patients. For this approach to work, it will probably be necessary to initiate immunotherapy before the first lower respiratory tract infection is established. The high levels of anti-Pseudomonas antibodies in the serum of infected CF patients apparently do little or nothing to enhance clearance of the pathogen. Appropriate vaccine candidates should be 1) broadly reactive with multiple *P. aeruginosa* isolates, 2) directed against surface exposed epitopes, and 3) opsonic. MEP and proteins F and H2 appear to satisfy all these criteria. In addition, all three antigens are expressed by *P. aeruginosa* isolates grown *in situ* in the CF lung. Further investigations with these immunogens are in progress.

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