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Antibodies to Cell Envelope Proteins of *Pseudomonas aeruginosa* in Cystic Fibrosis Patients

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Many vaccines containing somatic and secreted antigens of *Pseudomonas aeruginosa* have been reported. The vaccines containing lipopolysaccharide have been found to provide type-specific protection, but the endotoxin content of these vaccines does not make it feasible to use them in patients who are already debilitated. Outer membrane proteins could be effective as vaccines, as they can be purified free of lipopolysaccharide, and also because they are common to all serotypes of *P. aeruginosa*. To be effective as a vaccine, such proteins must be immunogenic and accessible from the outside of the intact bacterial cell. In this study, we showed that systemic antibodies were produced frequently to two cell envelope proteins with masses of 58,500 and 37,500 daltons and occasionally to the 34,000-dalton protein of *P. aeruginosa* in cystic fibrosis patients with chronic lung infections. In rabbits immunized with whole, fixed cells of *P. aeruginosa*, antibodies were also produced against the 58,500-dalton protein. Thus, the 58,500-dalton cell envelope protein of *P. aeruginosa* was the only immunogenic protein that was accessible to the immune system when whole, fixed cells were used for immunization. These serum antibodies did not protect the cystic fibrosis patients against further lung infection with *P. aeruginosa*.

Chronic lung infections caused by *Pseudomonas aeruginosa* are a major cause of morbidity and mortality in cystic fibrosis patients (1). The organisms remain localized in the lungs and seldom invade the blood stream in these patients (1). Antibodies against a variety of somatic and extracellular antigens of *P. aeruginosa*, such as antilipopolysaccharide antibodies and antitoxin, are found in the serum of these patients (9, 13, 15), but they do not afford any protection against repeated infection with *P. aeruginosa* (9).

Several antigens have been used experimentally as vaccines to protect against pseudomonas infections (2). A multivalent lipopolysaccharide (LPS) vaccine containing LPS from several serotypes has been used in cystic fibrosis patients in a small trial (13). Although a rise in serum antibody was achieved, no clinical or microbiological improvement was noted. Vaccines containing other antigens, such as ribosomes (11) and high-molecular-weight polysaccharide (14), are currently being investigated. Most of these antigens give type-specific protection and are therefore dependent on their LPS content. Recently, considerable interest has been shown in the use of certain major outer membrane proteins as vaccine antigens to protect against other bacterial diseases (10, 16). These major outer membrane proteins are common to all members of the species (A. L. Feldman, C. C. Dietz, and P. B. Fernandes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C214, p. 345) and, therefore, a monovalent vaccine may suffice to protect against all strains of *P. aeruginosa*. In addition, outer membrane proteins in the *Enterobacteriaceae* have been shown to cross-protect against other genera within the family (7). Another advantage of outer membrane proteins is that they do not have the toxic properties of LPS (10).

We have been interested in developing an aerosol vaccine containing major outer membrane or porin proteins of *P. aeruginosa*. For such a vaccine to be protective, the antigen must be immunogenic and accessible to the immune system. In this study, we have determined which cell envelope proteins are immunogenic when whole cells fixed in formaldehyde are injected into rabbits. We have also identified the cell envelope proteins to which antibodies are made in natural infections in cystic fibrosis patients.

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MATERIALS AND METHODS

Organism. *P. aeruginosa* strain 119, isolated from a cystic fibrosis patient, was used in these studies. The characteristics of this strain have been described previously (3). The organisms were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 12 h under aerobic conditions.

Immunization of rabbits. Whole cells of *P. aeruginosa* 119 were washed in phosphate-buffered saline and killed and fixed by suspension in phosphate-buffered saline containing 0.3% formaldehyde. After overnight refrigeration, the cell suspension was washed twice in phosphate-buffered saline. A 0.5-ml amount of this preparation containing 4×10^8 cells was injected intramuscularly into four New Zealand white rabbits. The animals were immunized twice, with a 2-week interval between injections. Rabbits were bled 2 weeks after the second immunization, and the sera were stored at −70°C.

Cell envelope protein preparation. *P. aeruginosa* was grown in 1 liter of Trypticase soy broth containing 2 mCi of [^{35}S]methionine (L-[^{35}S]methionine, 935 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 8 h. The labeled cells were washed in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.6) and lysed by sonication. After sonication, 1 mg each of deoxyribonuclease and ribonuclease was added to 20 ml of lysate, and unlysed cells were removed by centrifugation at 5,000 × *g* for 15 min. The supernatant containing the cell envelopes was centrifuged at 250,000 × *g* for 1 h, and the pellet was washed twice in 0.020 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.6). The final pellet containing washed cell envelopes was suspended in 2% Triton X-100 containing 10 mM ethylenediaminetetraacetic acid (EDTA), and the suspension was incubated at 30°C for 20 min. The suspension was again centrifuged at 250,000 × *g* for 1 h at 15°C. The supernatant containing solubilized cell envelope proteins was collected and dialyzed against phosphate-buffered saline (pH 7.2) for 24 h and diluted to contain 6 mg of protein per ml.

Immunodiffusion. Double diffusion was performed in 0.9% agarose in phosphate-buffered saline on glass slides. Rabbit sera were tested against the solubilized cell envelope preparation. Center wells were filled with undiluted antigen, and peripheral wells were filled with twofold dilutions of the rabbit sera.

Patient sera. Sera from 75 patients at St. Christopher's Hospital for Children, Philadelphia, Pa., were collected and coded with numbers (see Table 1). The patients belonged to different age groups ranging from 1 month to 30 years. Most of the patients had cystic fibrosis, but several had other respiratory diseases, such as viral pneumonia, chronic respiratory distress, and asthma. These sera were used for the detection of antibodies to the cell envelope proteins of *P. aeruginosa*.

Single radial immunodiffusion. Single radial immunodiffusion was used to screen patient sera for antibodies to antigens in the cell envelope preparation. It was carried out exactly as described by Hofstra and Dankert (8). The solubilized cell envelope preparation was incorporated in the agarose. Wells were punched in the agarose and filled with 2.5 μl of patient or rabbit sera. The diameters of the precipitation rings were measured after 48 h, and those sera which produced a precipitation ring were selected for the detection of antibodies to specific cell envelope proteins by immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Immunoprecipitation. A 1-ml amount each of rabbit or patient serum which was found to produce a precipitation ring was mixed with 1 ml of [^{35}S]methionine-labeled, solubilized cell envelope preparation. The mixtures were refrigerated for 48 h, and the precipitate formed was collected by centrifugation at 2,500 × *g*. The precipitate was washed twice with phosphate-buffered saline.

SDS-polyacrylamide gel electrophoresis. The washed precipitates were dissolved in sample buffer containing 0.05 M tris(hydroxymethyl)aminomethanehydrochloride (pH 6.8), 5% (vol/vol) mercaptoethanol, 2% (wt/vol) SDS, and 10% (vol/vol) glycerol and boiled for 5 min before loading on gels. The SDS-polyacrylamide gels containing 12% acrylamide were prepared as described previously (4). The gels were dried and exposed to Kodak XR-5 X-ray film for 48 h. Only the labeled cell envelope proteins, if present in the precipitate, were detected by this procedure.

RESULTS

Resolution of cell envelope proteins in Triton-EDTA-solubilized antigen preparations. Cell envelope proteins solubilized by 2% Triton–10 mM EDTA were compared with total cell envelope proteins solubilized by 2% SDS and boiling to determine the efficiency of our solubilization procedure. As seen in Fig. 1, the quantity of each of the proteins in the Triton-EDTA-solubilized preparation was less than that found in preparations solubilized by SDS and boiling. However, all the cell envelope proteins were represented in the Triton-EDTA-solubilized preparations in the same proportions as those found in the SDS-solubilized preparations.

Antibody in rabbit sera. Serial dilutions of immune rabbit sera yielded precipitin titers of up to 1:256. The antigen in these precipitates could have been LPS or protein or both in nature, and multiple precipitin lines were obtained.

Resolution of cell envelope proteins precipitated by rabbit antisera. All four antisera obtained from immunized rabbits precipitated a protein with a mass of 58,500 daltons from the cell envelope preparation (Fig. 2). Preimmune sera obtained from the same rabbits did not yield any precipitate. Thus, when whole, Formalin-fixed cells of *P. aeruginosa* were used for the immunization of rabbits, only one protein, the 58,500-dalton protein, was exposed at the cell surface and was immunogenic.

Antibody in patient sera. Of 75 sera tested, 23 yielded precipitates with the solubilized cell

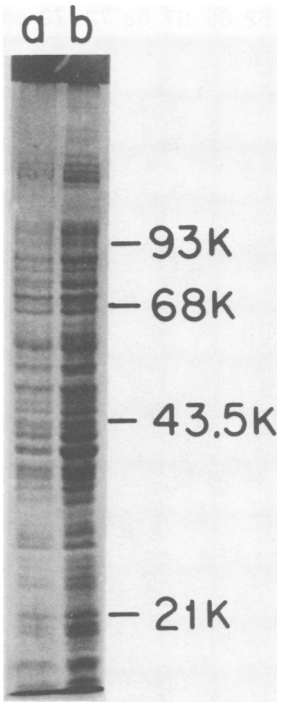

FIG. 1. *Resolution of cell envelope proteins solubilized with (a) 2% Triton-10 mM EDTA and (b) 2% SDS and boiling.*

envelope preparation in the single radial immunodiffusion assay. The diameters of the precipitates varied from 2.5 to 6.0 mm. Of the 23 patient sera, 21 were obtained from cystic fibrosis patients, and 2 were from patients with other respiratory diseases. Rabbit sera were used as controls. The immune rabbit sera had precipitation rings ranging from 5 to 7 mm in diameter. Sera from 39 patients with cystic fibrosis and 13 patients with other respiratory diseases did not produce any precipitation rings.

Resolution of cell envelope proteins precipitated by patient sera. As seen in Fig. 3, 13 of the precipitates contained a protein with a mass of 58,500 daltons. A total of 11 patients had antibodies to the 37,500-dalton major outer membrane protein in addition to the 58,500-dalton protein. One patient who did not have antibodies to the 58,500-dalton protein had antibodies to the 37,500-dalton protein alone. Six of the patients who had antibodies to the 58,500-dalton protein also had antibodies to the 34,000-dalton major outer membrane protein. These data are summarized in Table 1. Sera from nine patients who were positive in the single radial immunodiffusion test did not precipitate any of the cell envelope proteins. It is possible that these precipitates contained LPS-antibody complexes, and as they did not contain radioactive protein, they would not be detected by this procedure. Two of these patients had other respiratory illnesses and did not have cystic fibrosis. Occasionally, other faint bands were seen. As these were not repeatedly found in the precipitates, they were presumed to be non-specifically collected with the precipitate. Every patient who had antibodies to cell envelope proteins was diagnosed as having cystic fibrosis. Those cystic fibrosis patients who had antibodies to cell envelope proteins were between 7 and 30 years of age, whereas 16 patients with cystic fibrosis but without antibodies to cell envelope proteins were under 7 years of age. Every patient who had antibodies to the cell envelope proteins had recurrent pseudomonas lung infections. However, many other patients who had either a single pseudomonas lung infection or recurrent pseudomonas lung infections did not have serum antibodies.

The presence of serum antibodies to the cell envelope proteins did not appear to protect the patients against another lung infection by *P.*

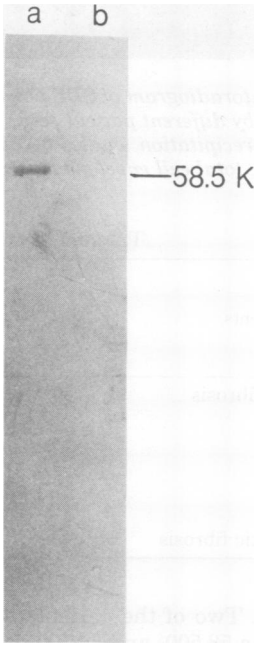

FIG. 2. *Autoradiogram of SDS-polyacrylamide gel showing cell envelope protein of P. aeruginosa precipitated by rabbit antisera prepared against Formalin-fixed organisms. (a) Rabbit antibody mixed with cell envelope proteins. (b) Normal rabbit serum mixed with cell envelope proteins. The cell envelope proteins were labeled with [^{35}S]methionine, and the labeled protein in the precipitate was demonstrated by autoradiography.*

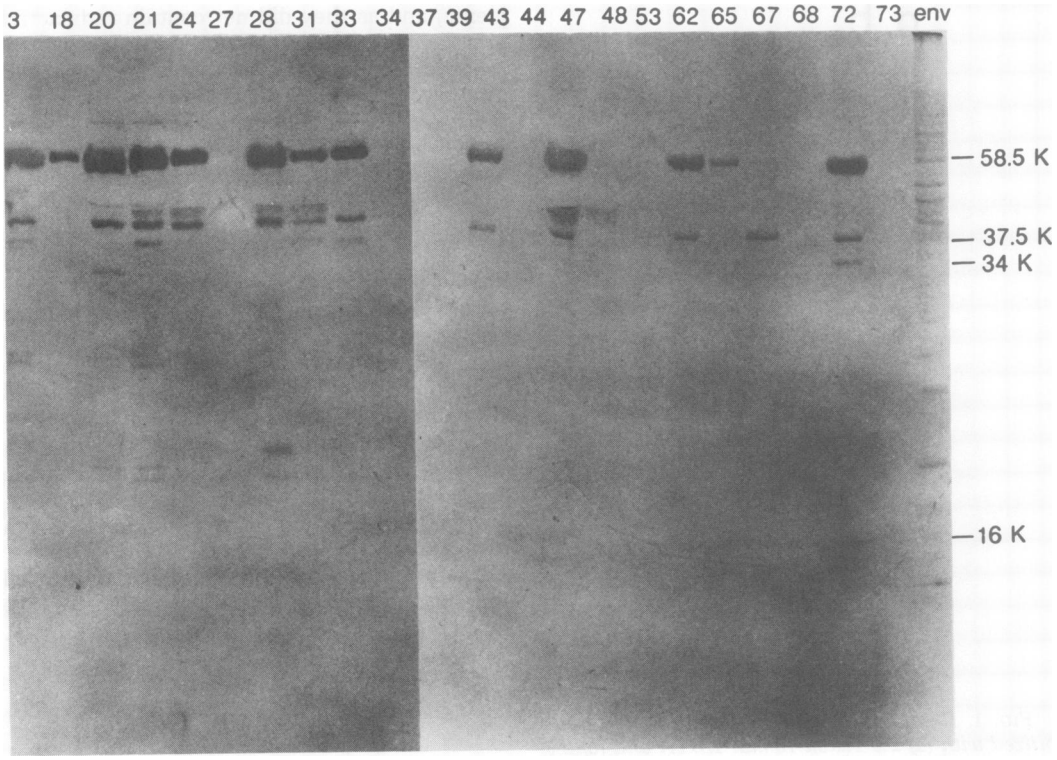

FIG. 3. *Autoradiogram of SDS-polyacrylamide gel showing [^{35}S]methionine-labeled cell envelope proteins precipitated by different patient sera. The numbers on the gel slots refer to patient sera which were selected for immunoprecipitation studies because they yielded precipitin rings in the single radial immunodiffusion test. Slot env, total cell envelope proteins.*

TABLE 1. *Distribution of antibodies to cell envelope proteins*

Patients	Total no. of patients tested	No. of sera positive by single radial immunodiffusion	Presence (+) or absence (−) of antibodies to cell envelope proteins			
			No. of sera	58,500 daltons	37,500 daltons	34,000 daltons
With cystic fibrosis	60	21	6	+	+	+
			5	+	+	−
			2	+	−	−
			1	−	+	−
			7	−	−	−
Without cystic fibrosis	15	2	0	−	−	−

aeruginosa. Two of the patients who had antibodies to the 58,500- and 34,000-dalton proteins and one patient who had antibodies to the 58,000-dalton protein are not living at the time of this report. *P. aeruginosa* was cultured from the sputa of these patients during their last hospital admission. At least five other patients with antibodies to cell envelope proteins have been reinfected with *P. aeruginosa*. Thus, the presence of serum antibodies to cell envelope proteins did not prevent reinfection of the lungs by *P. aeruginosa*.

DISCUSSION

The sera of cystic fibrosis patients have been shown to have antibodies against several somatic and extracellular antigens of *P. aeruginosa* (1, 9, 15). Although the development of these serum antibodies to *P. aeruginosa* antigens late in the disease may not be helpful against lung infec-

tions, it is possible that vaccination early in the disease, before the first infection with *P. aeruginosa* has occurred, may be of some benefit to the patient. In addition, it would be necessary to obtain high levels of secretory antibodies in the lungs to protect against chronic lung infections.

LPS vaccines have not been found to be useful against pseudomonas infections in adult cystic fibrosis patients (1, 13). Currently, many investigators are interested in major outer membrane protein (porin) vaccines against other bacterial pathogens (10, 16, 17). Such a vaccine against pseudomonas organisms would be useful as it would be less toxic because of its low endotoxin content and also because it might provide protection against all serotypes of *P. aeruginosa*.

The cell envelope proteins of *P. aeruginosa* have been characterized by other investigators (5, 6, 12). For an effective vaccine to be developed, the membrane protein must be immunogenic and exposed at the cell surface. In the past, it was not known which, if any, of the outer membrane proteins were exposed at the cell surface in whole cells and were immunogenic.

In this study, we found that antibodies were only made against a 58,500-dalton cell envelope protein when whole, fixed cells were used for immunizing rabbits. In many cystic fibrosis patients who had antibodies against cell envelope proteins, antibodies were produced against the 58,500-dalton protein. In addition, many of these patients also had antibodies against the 37,500-dalton major outer membrane protein. A few patients had antibodies to the 34,000-dalton protein. We do not know if repetitive immunization of rabbits would expand the spectrum of antibodies directed against the surface proteins.

Although Triton-EDTA does not release all of the cell envelope proteins quantitatively, in initial studies we found that all of the cell envelope proteins were represented in our cell envelope preparation. The 58,500-dalton protein is not a major cell envelope protein. We have not identified it as an outer membrane protein, but as antibodies were produced to it in whole, fixed cells, it is possible that it is an outer membrane protein. The 37,500- and 34,000-dalton proteins have been shown by other investigators to be two of the major outer membrane proteins (12). As the 58,500-dalton protein was selectively immunogenic when whole, fixed cells were used for immunization or in many natural lung infections, it may be beneficial to include the 58,500-dalton and possibly the 37,500- and 34,000-dalton proteins in cell envelope protein vaccine preparations.

The detection of antibodies to cell envelope proteins was correlated with the clinical condition of the patient at the time blood was drawn. It is not known why all patients with *P. aeruginosa* lung infections did not have antibodies to the 58,500-dalton protein. As patients under 7 years of age did not have serum antibodies to cell envelope proteins, it is possible that these proteins are less immunogenic or that they are masked by LPS, and thus antibodies are produced only by repeated infections. The patients with antibodies to cell envelope proteins were not protected against lung infections by *P. aeruginosa*. As the antibodies we detected were in the patients' sera, it is necessary to repeat these experiments with bronchial washings to determine if the antibodies are present at the site of infection. If present in the lungs, they may protect the patient against further lung infections. It is also not known whether antibodies to these proteins would prevent lung infections early in the disease, before damage to the lungs has occurred. It is possible that, to be effective, the antigens would have to be delivered locally to the lungs in such a manner as to result in the production of secretory antibodies to these cell envelope proteins.

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