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Quantitation and Identification of Antibodies to Outer-Membrane Proteins of *Pseudomonas aeruginosa* in Sera of Patients with Cystic Fibrosis

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Chronic, overwhelming pulmonary infection with *Pseudomonas aeruginosa* is a frequent problem in patients with cystic fibrosis. Titers of antibody to the outer-membrane proteins of *P aeruginosa* were 10^1–10^8 (as measured by an enzyme-linked immunosorbent assay) in the sera of 32 patients with cystic fibrosis. Fifteen patients who had been colonized with *P aeruginosa* for 18 months to nine years had a geometric mean antibody titer of 1.3×10^5—a value ~500-fold higher than that for 13 patients with cystic fibrosis who had never been colonized or for 16 healthy adults without cystic fibrosis ($P < 0.001$). A significant correlation was observed between the presence of antibody to outer-membrane proteins and the presence of antibody to mucoid exopolysaccharide ($P < 0.002$). Nineteen serum specimens from the patients with cystic fibrosis were allowed to react with Western electrophoretic blots of separated outer-membrane proteins. All of these sera contained antibodies to porin protein F. In addition, antibodies to outer-membrane proteins E, H2, and I and to a variety of minor protein components were observed in many sera.

Pseudomonas aeruginosa is commonly associated with progressive pulmonary disease in patients with cystic fibrosis (CF) [1]. Once established, colonization is rarely eliminated. *P aeruginosa* bacteremia virtually never occurs in these patients. To gain a better understanding of this peculiar host-parasite interaction, a number of investigators have studied the humoral immune response of patients with CF to *P aeruginosa* antigens. Hoiby and Axelsen [2] demonstrated by crossed immunoelectrophoresis that chronically colonized patients developed precipitating antibodies in their sera, with as many as 22 distinct specificities. These authors made no attempt to identify the antigens. Hoiby later observed [3] a positive correlation between the severity of infection and the number of precipitins. Other authors have reported fewer different types of antibody [4–8], but it has generally been concluded that patients with CF are capable of mounting an immune response to *P aeruginosa* antigens. Such patients may have serum antibodies to exotoxin A [9, 10], protease [10], mucoid exopolysaccharide [11–13], or three uncharacterized cell-envelope proteins [14] with molecular weights of 58,500 (possibly flagellin [15]), 37,500, and 34,000, respectively.

Using both polyclonal and monoclonal antibodies, we have demonstrated that the outer-membrane proteins of *P aeruginosa* are strongly conserved [15, 16]. For example, polyclonal antibodies to proteins E, F, H2, and I react with all 17 serotypes included in the *P aeruginosa* International Antigen Typing Scheme [15]. Furthermore, monoclonal antibodies to proteins F and H2 react with all 17 serotype strains as well as with 16 isolates from patients with CF [16, 17]. In the present study we demonstrate that patients with CF who are colonized with *P aeruginosa* have substantial titers of antibody to outer-membrane proteins, and we identify some of the individual outer-membrane proteins to which these patients respond.

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Informed consent was obtained from all patients in this study. The guidelines for human experimentation of the University of British Columbia and the Medical Research Council of Canada were followed in the conduct of this research.

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Materials and Methods

Antigen isolation. *P aeruginosa* PAO1 strain H103 was used for the isolation of outer mem-

branes by the one-step procedure of Hancock and Carey [18]. Lipopolysaccharide (LPS) was isolated from strain H103 by the technique of Darveau and Hancock [19].

Antisera and antibodies. Sera were collected from 32 patients with CF and from 21 healthy adults; the latter group of specimens included 15 individual serum samples from volunteers at the Vancouver Red Cross and pooled sera from six laboratory volunteers who routinely handled strain H103. After informed consent had been obtained, venous blood was drawn from all donors and was allowed to clot at 23 C for 45 min. The specimens were removed and stored frozen at −70 C without an apparent reduction of antibody titer. Of the 32 patients with CF whose sera were analyzed, 13 had no history of colonization by *P aeruginosa;* their average age was 9.1 years (range, seven months to 26 years). Four patients (aged three, seven, 18, and 19 years) were first found to be colonized when blood was drawn for the present study. Fifteen patients (average age, 13.1 years; range, five to 24 years) had been colonized with *P aeruginosa* for 1.5 to nine years.

The antibodies used as positive controls or for the identification of specific bands on Western immunoblots included the following monoclonal antibodies: MA1-8, specific for the LPS O-side chain of *P aeruginosa* serotype 5 [16]; MA3-5, specific for the rough core of the LPS of *P aeruginosa* serotypes 5, 7, 9, 10, 14, and 16 [17]; MA4-2, specific for protein F [17]; and MA1-6, specific for protein H2 [16].

Titration of antibodies. Antibodies were titrated by ELISA, with either outer membranes or LPS from strain H103 as the coating antigen; the ELISA was done as described previously [15, 16] except that dilutions of human serum in PBS were used as the first antibody and affinity-purified goat antibody to human IgA, IgG, and IgM (heavy and light chains) coupled to alkaline phosphatase (catalogue no. 15-10-07; KPL, Gaithersburg, Md) was used as the second antibody. A positive control, in which MA1-8 was used as antibody and outer membranes or LPS from strain H103 was used as antigen, was included on each microtiter plate. As negative controls, each serum was tested without precoating of ELISA wells with antigen; in this control system, background absorbance readings were obtained. Background responses were similar after the omission of serum or the second antibody from the ELISA. All sera were tested two to four times with consistent results. The ELISA titer was taken as the reciprocal log dilution of serum giving an absorbance value at 490 nm that was 30% of the positive control value. Absorbance was measured with a Titertek® Multiscan (Flow Laboratories, Mississauqua, Ontario) 2 hr after the addition of enzyme substrate. Control experiments demonstrating that the amount of antigen added was not limiting were also performed.

Electrophoretic blotting procedures. Outer-

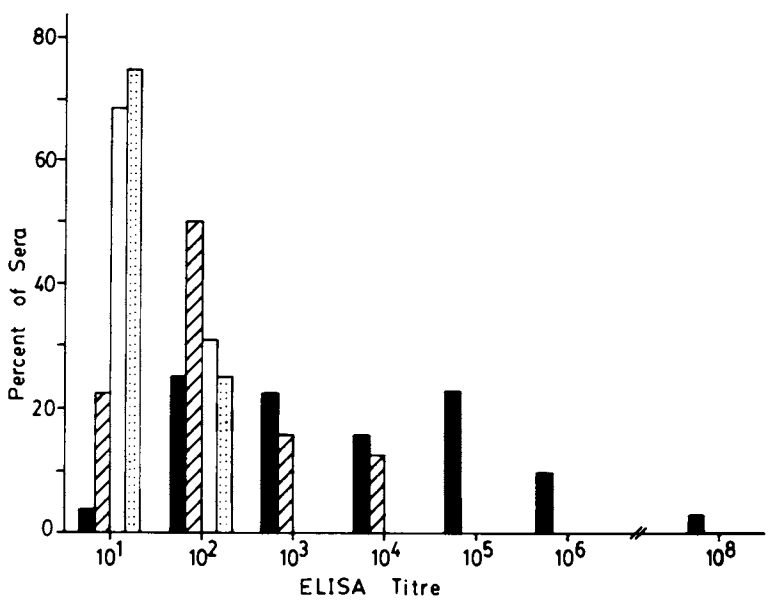

Figure 1. ELISA antibody titers to outer membranes and LPS from *P aeruginosa* strain H103 in the sera of 16 healthy adults and 32 patients with CF. Outer-membrane antibody titers in healthy adults and patients are indicated by filled and clear bars, respectively; LPS antibody titers in the two groups are represented by striped and stippled bars, respectively.

Table 1. Epidemiologic comparisons of ELISA antibody titers against outer-membrane antigens of *P aeruginosa* strain H103 in sera from patients with CF.

Epidemiologic group,* description (no. of patients)	Geometric mean ELISA titer†	χ^2,‡ *P*
A		
All patients with CF (32)	5.0×10^3	. . .
Controls (16)	2.5×10^1	30.0, <0.001
B		
Patients with CF not colonized by *P aeruginosa* (13)	2.5×10^2	. . .
Patients with CF initially found to be colonized when donating sera (4)	6.3×10^2	1.07, >0.5
Patients with CF colonized for 1.5–9 years (15)	1.3×10^5	20.7, <0.001
C		
Patients with CF and low titers of antibody to mucoid exopolysaccharide (7)	3.9×10^2	. . .
Patients with CF and high titers of antibody to mucoid exopolysaccharide (10)	1.6×10^5	17.0, <0.002

* The same antibody titration data served as a basis for all epidemiologic comparisons. In group C, only sera that had been screened by ELISA for antibody to mucoid exopolysaccharide [11] were considered. A high titer of antibody to mucoid exopolysaccharide was defined as a reading (OD_{490}) of 0.1–0.8 when the ELISA plate was coated with purified mucoid exopolysaccharide and all sera were diluted 1:32 with PBS. A low titer was defined as a reading of <0.01. Only patients with CF were considered in groups B and C.

† Because of the wide range of individual titers covering seven orders of magnitude, geometric mean values were used for assessment of the data.

‡ χ^2 values are the results of statistical analysis by construction of contingency tables.

membrane components were separated on 14% acrylamide SDS-polyacrylamide gels after solubilization at 100 C for 5 min, as previously described [18]. These components were then transferred to nitrocellulose by the method of Towbin et al [20] and were allowed to react in turn with the following: fetal calf serum, which blocked nonspecific protein-binding sites on the nitrocellulose; a dilution of human serum that gave an ELISA titer of 10^2–10^3; an affinity-purified goat antiserum to human whole immunoglobulin coupled to alkaline phosphatase; and an alkaline-phosphatase histochemical substrate, Fast Red TR salt and Naphthol AS-MX phosphate; these experiments were performed essentially as described previously [15, 17]. Control experiments for the identification of the specific outer membrane–protein bands included incubation of individual blots with monoclonal antibodies MA1-8, MA3-5, MA4-2, and MA1-6 (to reveal the position of smooth LPS, rough LPS, protein F, and protein H2, respectively) and amido-black staining of blots of outer membranes and of electrophoretic blots of molecular-weight standards (to reveal the positions of other components).

Statistical analyses. All data were analyzed pairwise by the construction of contingency tables (usually 2 × 4 or 2 × 6). The data were then subjected to a χ^2 analysis for independence of row and column classifications; a calculator (model TI58; Texas Instruments, Houston) fitted with an applied statistics module was used.

Results

ELISA titers to outer-membrane components. Figure 1 shows data obtained from the titration of sera from 32 patients with CF against outer membranes from *P aeruginosa* strain H103. Titers of antibody specific for outer membranes (that is, proteins and LPS) ranged from 10^1 to 10^8, with a geometric mean titer of 5.0×10^3. We also titrated sera against LPS from strain H103 to demonstrate that the titers measured were largely due to reactions against outer-membrane proteins and not by a reaction with LPS. The reactions of the sera against LPS from strain H103 were invariably weaker than those against outer membranes (proteins and LPS) ($\chi^2 = 18.6$; $P < 0.01$); the geometric mean ELISA titer against LPS was 150. This result does not imply that sera from patients with CF react poorly with LPS, since LPS from *P aeruginosa* serotype 5 (represented by strain H103) is relatively uncommon among clinical isolates from patients with CF [21]. Control sera (figure 1) responded significantly more weakly ($\chi^2 = 30.0$; $P < 0.001$) than sera from patients with CF to outer membranes from strain H103 (geometric mean titer, 23) and somewhat more weakly ($\chi^2 = 9.0$; $P < 0.05$) to LPS from this strain (geometric mean titer, 17). A pool of sera from laboratory volun-

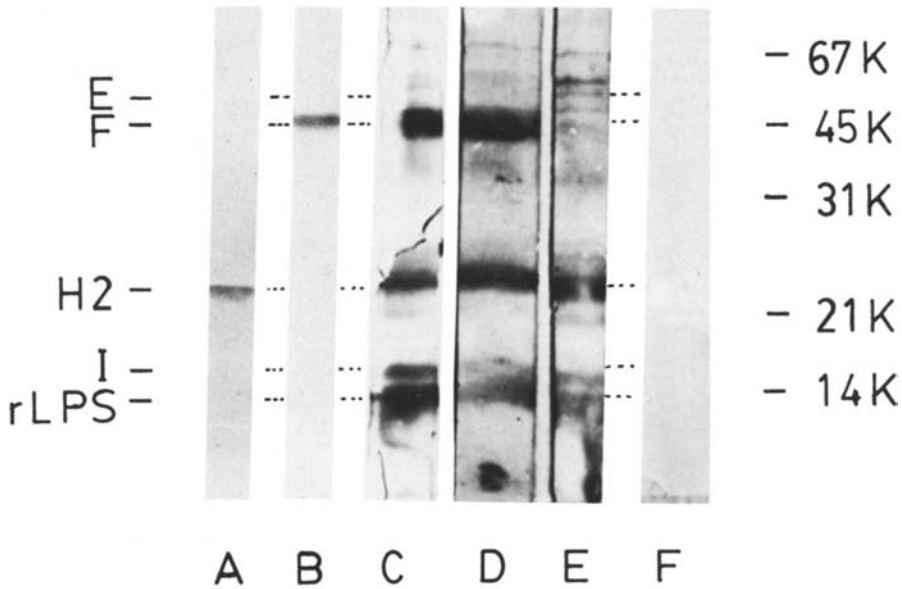

Figure 2. Western electrophoretic blots of separated outer-membrane components of *P aeruginosa* strain H103 after interaction first with human serum or mouse monoclonal antibodies and then with goat antiserum to human immunoglobulin conjugated to alkaline phosphatase and a histochemical stain for alkaline phosphatase. Lanes A and B: Outer membranes were allowed to react with monoclonal antibodies MA1-6 (specific for protein H2) and MA4-2 (specific for protein F), respectively. Lanes C, D, and E: Sera CF22, CF9, and CF8, respectively, were used as the first antibody. (All of these sera were from patients with CF.) Since the level of the background reactions in these lanes was high (because of the wide range of antibodies present in the sera), weaker bands were lost during photographic reproduction. Lane F: Serum from a healthy adult volunteer was used. The running position of molecular-mass standards (in kilodaltons) is indicated to the right of the gel, and outer membrane components are designated on the left.

teers who routinely handled strain H103 contained low titers of antibody (10^1) to both outer membranes and LPS; this finding demonstrated that contact with *P aeruginosa* was not in itself sufficient to cause elevated serum antibody titers.

When various epidemiologic factors were considered, a highly significant correlation ($P < 0.002$) was found between the presence of antibodies to outer-membrane antigens of *P aeruginosa* in the patients' sera and (*1*) documented colonization for more than one year by *P aeruginosa* and (*2*) the presence, as determined in a previous study [11], of serum antibodies to mucoid exopolysaccharide (table 1). When only those patients known to be colonized with *P aeruginosa* were considered, geometric mean ELISA titers to outer membranes (1.3×10^5) were found to be much higher than titers to LPS (5.4×10^2) ($\chi^2 = 11.6$; $P < 0.01$). This result again suggested that the antibodies being measured against whole outer membranes were directed primarily against outer-membrane proteins rather than against LPS. However, titers of antibody to LPS were significantly higher ($\chi^2 = 15.0$; $P < 0.01$) in sera from colonized patients (mean titer, 5.4×10^2) than in those from uncolonized patients (mean titer, 35) or controls (mean titer, 17). Presumably, these relatively low titers against LPS from strain H103 represented antibodies to common determinants in the LPS.

The presence of high levels of antibody to outer-membrane proteins in patients with CF who are chronically colonized with *P aeruginosa* suggests that this antibody is ineffective against pulmonary disease in patients with CF. Furthermore, these data suggest that immunization with outer membrane proteins would be of little value once chronic colonization by *P aeruginosa* was established in patients with CF. However, the possibility remains that immunization prior to colonization could be an effective preventive strategy. Protein F, which we have shown to be a common antigen in the strains of *P aeruginosa* studied so far [15] and to be surface exposed [17], is clearly antigenic in humans (figure 2) and would be a reasonable candidate for a monovalent outer membrane-protein vaccine.

While this is the first definitive report of antibodies to specific outer-membrane proteins, Fernandes et al [14] used immunoprecipitation to demonstrate antibodies to two cell-envelope (outer-plus-inner membrane) proteins of 58,500 daltons and 37,500 daltons in the sera of 13 of 60 patients with CF. However, these researchers did not characterize the specific location of these proteins in the bacterial cell envelope. This point is probably important, since we have recently shown by crossed immunoelectrophoresis that many precipitins in the sera of patients with CF are not directed against outer-membrane proteins [22]. Indeed, although the 58,500-dalton protein was the major polypeptide precipitated by CF sera in the study of Fernandes et al [14], no major outer-membrane protein of this molecular mass exists in *P aeruginosa* [18]. We have proposed that this polypeptide is flagellin [15], which, although attached to the cell envelope, is not a cell-envelope protein. Despite the slight contamination of the outer membranes of strain H103 by flagellin [15], we observed only small amounts of antibody to flagellin in sera from patients with CF in the present study. In contrast, a rabbit hyperimmunized with outer membranes had large quantities of antibody to flagellin [15].

We recently showed that a large proportion of *P aeruginosa* isolates from patients with CF are nontypable by the Fisher typing scheme, serum sensitive, and deficient in LPS O-side chains [23]. We postulated that the serum susceptibility of these strains may explain in part why pseudomonas bacteremia is rare among patients with CF. The high titers of circulating antibodies to outer-membrane components could augment the bactericidal capacity of serum via the classical complement pathway. These antibodies could also function as opsonins, thereby enhancing phagocytosis and clearance of *P aeruginosa* organisms that gain access to the bloodstream.

Characterization of antibodies in sera from patients with CF. To obtain information about the presence of antibodies to individual outer-membrane proteins in the sera of patients with CF, we studied the interactions of these sera with Western electrophoretic blots of outer-membrane proteins. As discussed previously [15], not all of the proteins were transferred quantitatively from SDS-polyacrylamide gels to nitrocellulose. In addition, the proteins were present in different amounts in the outer membranes. These two facts, together with the presumably different affinities of individual antibodies for their respective antigens, meant that we could not make quantitative conclusions about the amounts of specific antibodies in the sera. However, by use of monoclonal antibodies to specific outer-membrane proteins and by cotransfer of molecular-weight standards with outer-membrane proteins from slab gels to nitrocellulose and subsequent amido-black staining of both, we were able to draw precise conclusions about the specificities of some of the antibodies in the sera.

Of the 32 sera titrated by ELISA, only the 18 with the highest titers and serum CF17 were examined by this method. Representative data are shown in figure 2, lanes C, D, and E. All 19 sera reacted with protein F (table 2), and most reacted with protein H2 (78%) and the rough core of LPS (74%). In addition, more than 50% of the sera re-

Table 2. Specific antibodies to outer-membrane antigens of *P aeruginosa* strain H103 in sera of patients with CF.

Serum	ELISA titer to whole outer membranes	Antibodies to indicated antigens identified on blots*						
		50 kd†	E	F	21–35 kd†	H2	I	LPS
CF8	10^5	+	+	+‡	+	+	+	+
CF9	10^8	+	+‡	+	+	+	+	+
CF10	10^6	+	+	+	+	+	+	+
CF12	10^4	–	–	+	–	+	–	–
CF14	10^5	–	–	+	+	–	+‡	–
CF17	10^2	–	–	+	–	+	–	+
CF19	10^5	+	+	+	+	+	–	+
CF20	10^3	–	–	+	–	+	+	+
CF22	10^5	+	+	+	+	+	+	+
CF24	10^4	–	–	+	+	+	–	+
CF26	10^6	–	–	+	+	+	–	+
CF27	10^5	–	–	+	–	+	–	–
CF28	10^4	–	–	+	–	+‡	+	+
CF32	10^3	+	+	+‡	–	+‡	–	+
CF36	10^5	+	+	+	+	+	–	+
CF37	10^4	+	–	+	–	–	–	–
CF39	10^3	+	+	+	+	–	–	–
CF40	10^6	+	+	+	+	+	–	+
CF42	10^5	+	–	+	–	–	–	+

* + = identifiable band; – = no observed reaction.

† kd = kilodaltons; >50 kd and 21–35 kd refer to molecular-mass regions of the Western electrophoretic blots rather than to specific proteins. A variety of minor outer-membrane proteins were responsible for the observed positive reactions (figure 2).

‡ This reaction was weakly positive, producing only faint bands.

acted with a variety of high-molecular-weight polypeptides, protein E, and a variety of polypeptides of 21–35 kilodaltons (table 2). Four sera from healthy volunteers apparently did not react with individual outer-membrane components on electrophoretic blots; the results with one of these sera are shown in figure 2, lane F.

Discussion

These studies demonstrated that the outer membrane proteins of *P aeruginosa* are antigenic in patients with CF, since antibodies to a variety of individual outer-membrane proteins were found in the sera of such patients. The mean ELISA antibody titer against outer membranes in all patients with CF was 5×10^3, whereas the titer in patients with CF who were chronically colonized with *P aeruginosa* was $>10^5$. By way of comparison, rabbits hyperimmunized with outer membranes of *P aeruginosa* strain H103 (four injections of 50 μg at two-week intervals) had ELISA titers to heterologous outer membranes of $\sim 10^5$–10^6 [15].

The data in the present study confirm earlier observations that the presence of circulating antibodies to *P aeruginosa* is an indicator of chronic respiratory disease in CF [2–14] (table 1) and that the extent of the immune response is related to the length of colonization with *P aeruginosa* [2, 3, 11] (table 1). The four patients with CF who were first found to be colonized with *P aeruginosa* when their blood was drawn for the present study had titers of antibody to outer-membrane antigens comparable to those in patients with CF who had never been colonized (table 1). Thus, chronic colonization apparently must be established before patients with CF mount a serum antibody response to *P aeruginosa*. A major unanswered question raised by this and similar studies is why patients with CF are unable to eradicate *P aeruginosa* despite such high levels of antibody to cell-surface antigens.

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