JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1989, p. 2789–2793
0095-1137/89/122789-05$02.00/0

Vol. 27, No. 12

Pseudomonas aeruginosa Flagellar Antibodies in Patients with Cystic Fibrosis

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An enzyme-linked immunosorbent assay specific for flagellum type (a or b) of *Pseudomonas aeruginosa* was used to detect serum immunoglobulin antibodies in 98 random outpatients and 14 colonized cystic fibrosis patients. Antibodies were detected to both types of flagella in addition to M-2 lipopolysaccharide. Titers to both flagellar antigens (FlAg) were 10 to 100 times higher in cystic fibrosis patients than in random outpatients of a comparable age group. Mean antibody titers against b-type FlAg were 454 for outpatients (ages newborn to 21 years), whereas the mean titer for cystic fibrosis patients (ages 6 to 21 years) was 51,520. Titers against a-type FlAg were generally lower, with mean outpatient titers of 68 and mean cystic fibrosis patient titers of 34,323. Differences were also seen in antibody titer against M-2 lipopolysaccharide, but these differences did not correspond to M-2 FlAg titers. In 98 random outpatients (ages newborn to 86 years), FlAg titers generally increased with age. To demonstrate further specificity of the enzyme-linked immunosorbent assay for flagellum antibody, Western blots were performed with selected high-titer cystic fibrosis patient sera. Sera that had a high titer (>25,600) for b- or a-type FlAg showed a corresponding reactive band. These results demonstrate that flagellum antibodies are produced in humans in response to *P. aeruginosa* infection.

Pseudomonas aeruginosa is frequently a pulmonary pathogen of patients with cystic fibrosis (CF) (8, 24). Chronic lung infection often results and is one of the major causes of death in these patients (25). Antibodies to a variety of *P. aeruginosa* surface antigens, including phospholipase C (14, 15), exotoxin A (14, 15), alkaline protease (10, 14, 15), lipopolysaccharide (LPS) (13), alginate (6), and outer membrane proteins (5), have been demonstrated in CF patients.

P. aeruginosa flagella are classified as two types, designated a and b, based on slide agglutination assays and apparent molecular weight of the flagellin. Immunofluorescence (3) and, more recently, an enzyme-linked immunosorbent assay (ELISA) (17), have been used to discern the two flagellum types. The b types comprise a homogeneous group with a flagellin molecular weight of 53,000. The heterogeneous a types can be divided into several subgroups consisting of flagellin molecular weight of 45,000 to 52,000 (1). The a-type flagella exhibit one to three of the antigen subtypes, a_1, a_2, a_3, a_4, but also appear to have a dominant antigen designated a_0.

Flagella of *P. aeruginosa* have been demonstrated to serve as virulence factors (12, 18). In a burned mouse model, nonflagellated, nonmotile mutants are significantly less virulent than flagellated, motile parental strains (12, 18). Motile organisms rapidly disseminated from the original burn wound, resulting in bacteremia, whereas nonmotile mutants were not invasive. Additionally, passive transfer of flagellar antiserum resulted in protection which was specific for the flagellar type (11). These data were consistent with results that showed that flagellum antibody was opsonic (2). Cross-protection was demonstrated among the heterogeneous a types, supporting evidence for the cross-reactive a_0 antigen. The cross-reactive a_0 antigen has also been demonstrated by using an ELISA (17) and by opsonophagocytosis assays (T. R. Anderson and T. C. Montie, Can. J. Microbiol., in press).

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This study was initiated to investigate whether *P. aeruginosa* flagellar antibodies were produced in colonized symptomatic CF patients as compared with random outpatients of similar age. Serum immunoglobulin G antibodies were quantitated by ELISA, and specific antibodies were verified by Western blotting (immunoblotting).

MATERIALS AND METHODS

Sera. Human serum samples were obtained from 98 random outpatients presenting for venipuncture. Patients ranged in age from newborn to 86 years, with specimens included from each decade of life. Sera from 14 CF patients colonized with *P. aeruginosa* were obtained from Children's Hospital National Medical Center, Washington, D.C. Normal human serum was prepared by pooling 0.2-ml samples of seven outpatient neonatal sera and adsorbing repeatedly with strains M-2 (b type) and 5940 (a type) whole cells as previously described (11).

Flagellar antigen (FlAg) and LPS preparation. *P. aeruginosa* SBI-N (a type), a clinical burn isolate, and M-2 (b type), a standard laboratory strain, were provided by I. A. Holder, Shriners Burn Institute, Cincinnati, Ohio. Strain 5940 was provided by R. Ansorg, Collection d'Institut Pasteur, Paris, France. Flagellar filaments were routinely prepared as previously described (1). Briefly, partially purified material was obtained by growth of bacteria in mineral salts medium, shearing, and differential centrifugations. Further purification involved a dual-column molecular seiving procedure with the addition of deoxycholate and EDTA to facilitate removal of LPS (19).

LPS was prepared from *P. aeruginosa* M-2 (IATS [International Antigenic Typing Scheme] O type 2,5) as previously described by Darveau and Hancock (7).

ELISA. The indirect ELISA was used to determine the presence and type of *P. aeruginosa* flagellum antibody in outpatient and CF sera. FlAg was diluted in carbonate buffer to a concentration of 200 ng/ml. FlAg (50 μl per well) was absorbed to a polyvinyl chloride microdilution plate (Fisher

Scientific Co., Pittsburgh, Pa.) by overnight incubation at 4°C. The plate was washed three times with phosphate-buffered saline (PBS)-Tween (0.8% NaCl, 0.8% KH_2PO_4, 0.12% Na_2HPO_4, 0.02% KCl, 0.05% Tween 20), and 100 μl of coating buffer consisting of PBS–3% gelatin was added to each well. After 45 min of incubation at 37°C, the plate was washed three times with PBS-Tween. Then 50 μl of CF serum diluted 1 in 200 or outpatient sera diluted 1 in 20 in PBS-Tween–1% gelatin was added, and 1:1 doubling dilutions were made to a final concentration of 1 in 102,400 for CF sera or 1 in 10,240 for outpatient sera. After a 60-min incubation at 37°C, the plate was washed, and 50 μl of goat anti-human immunoglobulin conjugated to peroxidase (Hy-Clone, Logan, Utah) diluted 1 in 10,000 in PBS-Tween–1% gelatin was added to each well. The plate was incubated at 37°C for 60 min and washed three times. Then 50 μl of the substrate (0.1 M citric acid [anhydrous], 0.2 M disodium phosphate [anhydrous], 1% *ortho*-phenylenediamine [Sigma Chemical Co., St. Louis, Mo.], and 0.03% H_2O_2 [pH 6.0]) was added to each well and allowed to develop at room temperature for 3 to 5 min. The reaction was stopped by the addition of 2.5 M sulfuric acid. The amount of substrate hydrolyzed was proportional to the amount of antibody present. The net A_{490} was read on an ELISA plate reader (Bio-Tek Instruments), and the titer was determined as the reciprocal of the last dilution in which the absorbance value was twice that of the adsorbed normal human serum control.

Western blots. FlAg was electrophoresed on homogeneous 12.5% polyacrylamide gels by using a PHAST system (Pharmacia Fine Chemicals, Piscataway, N.J.). Proteins were blotted onto nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell Co., Keene, N.H.) by diffusion and blocked with 3% bovine serum albumin in Tris-buffered saline for 2 h at 37°C. After a rinse, 10 ml of human serum diluted 1:20 in NETG buffer (0.85% NaCl, 0.19% EDTA, 0.75 Tris, 0.25% gelatin [pH 7.4]) was added to membranes and incubated at 37°C for 1 h with gentle shaking. After a wash with 200 ml of NETG buffer, 10 ml of goat anti-human immunoglobulin conjugated to peroxidase diluted 1:400 in NETG was added to membranes and incubated at 37°C for 1.5 h with shaking. Then 10 ml of substrate solution (1% orthophenylenediamine–0.03% H_2O_2 in Tris-buffered saline) was added after washing with 200 ml of NETG buffer. The reaction was allowed to develop at room temperature and was stopped by repeated rinsing with distilled water.

RESULTS

In Fig. 1a and b, random outpatient (ages 1 to 89 years) sera were assayed for flagellar antibodies. Both a- and b-type FlAg induced antibodies in the surveyed population. Some increase in response was observed in titer against b-type FlAg near age 20 years (Fig. 1a). Infants (<1 year), showed essentially no titer, but response increased with age, and higher titers up to 10,240 were observed. In comparison, lower titers were seen against a-type FlAg. With the exception of one patient, titers were 2,560 or below (Fig. 1b). Titers of <20 are considered negative for flagellum and LPS antibody by our criterion (absorbance of the beginning dilution is less than twice the absorbance of the beginning dilution of the negative control, adsorbed normal human serum). Therefore, 20, 13, and 64% of outpatients are negative for the presence of antibodies to M-2 FlAg, M-2 LPS, and SBI-N FlAg, respectively. A more direct comparison can be seen in Fig. 1c, which also includes data from M-2 LPS. The data were grouped to show the average titer

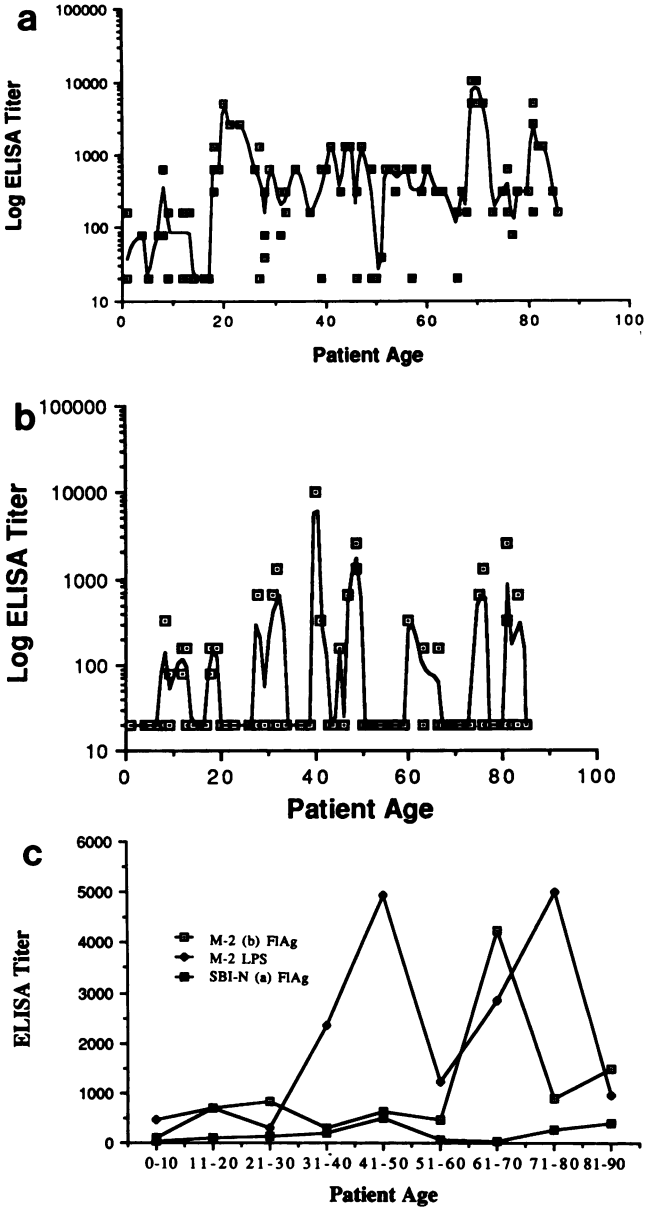

FIG. 1. (a) M-2 (b type) FlAg ELISA titers of random outpatient (aged newborn to 86 years) sera; (b) SBI-N (a type) FlAg ELISA titers of random outpatient (aged newborn to 86 years) sera; (c) ELISA titers against FlAg (a and b types) and M-2 LPS of random outpatient sera. Points are the average of titers for 10-year age groups.

for 10-year age spans. A varied profile between the b-type FlAg and its LPS was observed. The LPS response increased dramatically in patients aged 31 to 40 years and 61 to 70 years, whereas the b-type the FlAg had a similar increase only in the group aged 61 to 70 years. This variation in response was also seen with the individual patient sera. Only 3% of the patients showed multiple high titers (5,120 to 10,240) to both b-type FlAg and LPS (data not shown).

To ascertain to what extent flagellar antigen is capable of eliciting an antibody response, we tested 14 colonized and/or infected CF patients. In Fig. 2, 3, and 4, data from CF sera (patients aged 6 to 19 years) are plotted. A dramatic increase

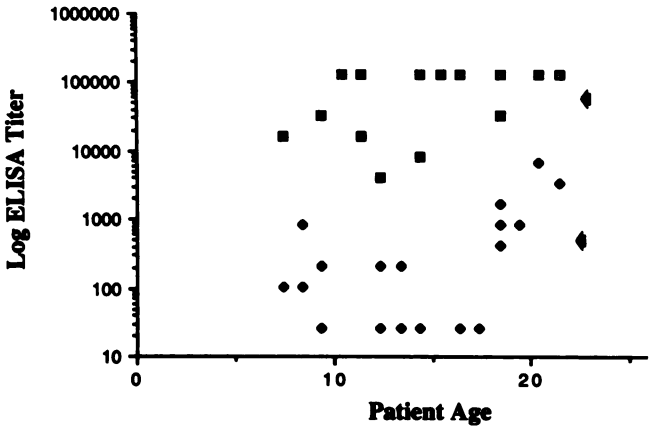

FIG. 2. M-2 (b type) flagellum antibody titers of CF (■) and random outpatient (◆) sera as determined in an ELISA. Significantly higher titers against M-2 flagella were seen in colonized CF patients when compared with those in random outpatients. Analysis by the Student *t* test gave significant *t* values beyond the 0.001 level. Arrows represent arithmetic means of CF and outpatient titers.

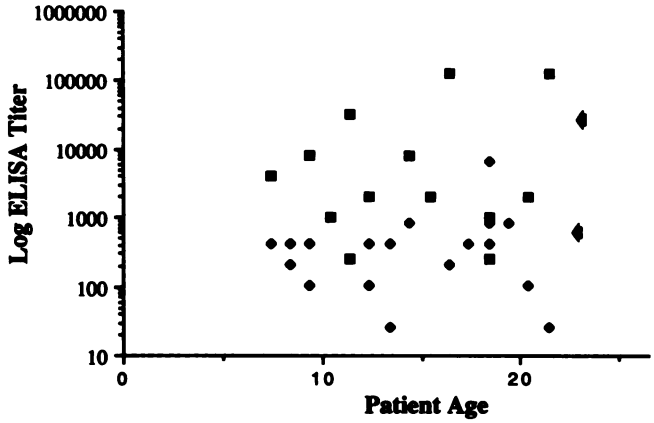

FIG. 4. M-2 LPS antibody titers of CF (■) and random outpatient (◆) sera as determined in an ELISA. Significantly higher titers against M-2 LPS were seen in colonized CF patients when compared with those in random outpatients. Analysis by the Student *t* test gave significant *t* values beyond the 0.01 level. Arrows represent arithmetic means of CF and outpatient titers.

in FlAg and LPS antibodies was seen. Type-b flagella gave antibody titers that averaged 51,520, compared with 454 for the same normal outpatient age group (Fig. 2). Upper and lower arrows indicate mean titers for CF patients and random outpatients, respectively. Mean titers against a-type flagella were 34,322 in CF patients compared with a mean titer of 68 for comparable normal outpatients (Fig. 3). M-2 LPS gave a mean titer of 18,542 for CF patients compared with 568 for comparable normal population (Fig. 4).

In Table 1, the results of Western blots and ELISA titers with CF and selected outpatient sera are shown. Maximum titers of 102,400 for b-type FlAg were obtained for 57% of the CF patients. In some patients, high titers were obtained to both FlAgs. These data indicated that infections with at least two different strains were involved or had occurred at some point in time. Sera that had a high titer (>25,600) in the ELISA reacted specifically with the corresponding FlAg in Western blots, whereas titers of less than 25,600 were not reactive. This correlation was true with CF sera; however, no outpatient serum had a high enough titer to give a positive blot.

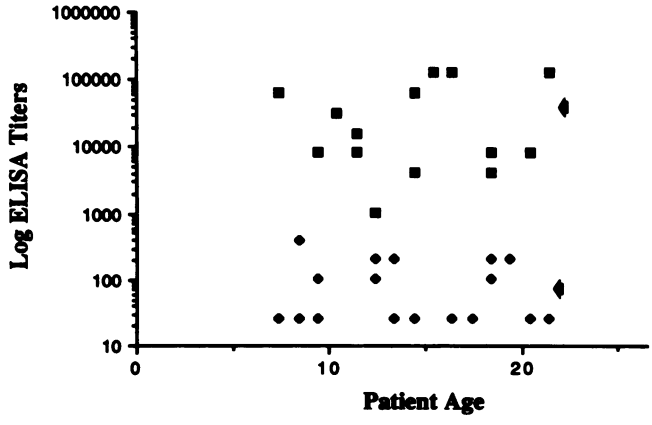

FIG. 3. SBI-N (a type) flagellum antibody titers of CF (■) and random outpatient (◆) sera as determined in an ELISA. Significantly higher titers against SBI-N flagella were seen in colonized CF patients when compared with those in random outpatients. Analysis by the Student *t* test gave significant *t* values beyond the 0.001 level. Arrows represent arithmetic means of CF and outpatient titers.

DISCUSSION

It is interesting that although clinical isolates are commonly represented by both a- and b-type strains, some CF patients gave strong antibody reactions to only one FlAg type (Table 1). These results suggest that these patients have harbored organisms with only one flagellum type and perhaps only one strain, although multiple infections with different strains containing the same FlAg type could occur. These observations of one flagellum type antibody are consistent with several recent reports that have employed DNA probes to a specific region upstream from exotoxin A

TABLE 1. ELISA titers and Western blot results of CF sera

Patient no.	Reactive band in Western blot (*n*)[a]	ELISA titer		
		M-2 (b type)	SBI-N (a type)	M-2 LPS
CF patients				
1	M-2 (2)	102,400	6,400	1,600
2	M-2, SBI-N (2)	102,400	102,400	1,600
3	SBI-N (1)	12,800	51,200	3,200
4	M-2 (2)	102,400	6,400	<200
5	ND[b]	12,800	12,800	200
6	None (2)	6,400	51,200	6,400
7	ND	3,200	800	1,600
8	M-2, SBI-N (2)	102,400	25,600	800
9	ND	25,600	3,200	800
10	M-2 (1)	102,400	3,200	6,400
11	M-2, SBI-N (2)	102,400	102,400	102,400
12	M-2, SBI-N (2)	102,400	102,400	102,400
13	M-2 (1)	25,600	6,400	6,400
14	M-2 (2)	102,400	6,400	25,600
Outpatients				
50	None (1)	640	10,240	10,240
79	None (2)	10,240	320	320
86	None (2)	160	<20	10,240

[a] *n*, Number of assays.
[b] ND, Not done.

or specific pilin genes. Colonization patterns were followed in certain CF patients (20–22). Based on the probe analysis, a general conclusion was that single-strain colonization was the norm, even though multiple antigenic phenotypes were represented in the single strain (20–22). On the other hand, from our data in some CF patients colonization with organisms with at least two different flagellum types occurred at some point. This latter result does not discriminate between sequential or multiple infections with respect to time.

Our past evidence showed that in severely and chronically colonized CF patients, deficiencies in flagellum motility and chemotaxis, as well as other invasive virulence factors, were observed in *P. aeruginosa* rough isolates (16). We hypothesized that invasive virulence factors were selected against in vivo but that initial colonization occurred with strains exhibiting invasive virulence factors, including the total flagellar regulon. The latter strains were associated with patients with less severe and primary colonization syndromes. The data presented here are consistent with this general hypothesis in that colonization with flagellated (invasive) strains had occurred, since high levels of flagellum antibody were detected in 93% of the tested patients.

We view the lower response to a-type antigen in outpatient and CF sera (Fig. 1c) as a reflection of decreased sensitivity of the a-type antibody in binding to FlAg at the level of antigen employed in the assay. Although the degree of sensitivity may be affected when low amounts of antigen are used in the ELISA (10 ng), this approach contributes to retaining specificity, since we obtained the same results with partially pure and purified preparations. Furthermore, our current interpretation is that the heterogeneous nature of the a-type flagella reduces the relative exposure of the common a_0 antigen and, therefore, that generation of specific a-type antibody also may be reduced.

Flagellar antibody responses shown by ELISA titers were consistent with results from Western blots. Although not as sensitive as electrotransfer methods, the diffusion method proved adequate in rapidly identifying the presence of specific flagellum antibodies in patients with high ELISA titers (25,600 or above). The position of the reactive band coincided with the gel band and the approximate molecular weight of the flagellin expected (Table 1). The presumed b-type band also coreacted with a b-type mouse monoclonal antibody, further verifying the identification (data not shown).

In general, low titers to both types of *P. aeruginosa* flagella and M-2 LPS were seen in random outpatients. Increases in antibody titers to flagella observed in random elderly outpatients remains to be explained but may reflect an increase in transient infections coupled to a decrease in effective clearance mechanisms. Titers to both a- and b-type flagella and LPS were significantly higher in CF patients than in random outpatients.

In conclusion, a population at high risk of pulmonary infection with *P. aeruginosa* developed significant flagellum antibodies as compared with age-appropriate outpatients. The high titers obtained to flagellar antibody suggest that it may be a good candidate marker for infection. Phospholipase antibody levels appear to be of similar value (15). We have shown that some selection mechanisms may favor emergence of an abnormally high number of FlAg variants, especially in chronically infected patients (16). The observation of flagellar antibody titers of 25,600 or greater in all patients indicates that FlAg was expressed in an early or intermediate stage in colonization. Anwar et al. have also indicated the presence of flagellar antigen on bacteria directly isolated from the sputum (4).

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Physicians' Medical Education Research Foundation, The University of Tennessee Medical Center.

We are particularly appreciative of the gift of CF sera provided through the efforts of Robert J. Fink and staff, Children's Hospital, Washington, D.C. Outpatient serum was kindly provided by Vincent McCarthy.

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