INFECTION AND IMMUNITY, Nov. 1985, p. 572–576
0019-9567/85/110572-05$02.00/0

Vol. 50, No. 2

Avirulence and Altered Physiological Properties of Cystic Fibrosis Strains of *Pseudomonas aeruginosa*

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Received 19 April 1985/Accepted 7 August 1985

Twenty *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis in good and poor clinical condition were typed by the American Scientific (Difco Laboratories, Detroit, Mich.) Typing Scheme. Only five strains were agglutinated with a single typing serum. Ten strains were agglutinated with more than one serum, and five were not agglutinated with any serum, suggesting some type of lipopolysaccharide alteration in the majority of these strains. Of the strains from patients in good clinical condition, 72% demonstrated proteolytic activity, while 60% of the strains from patients in poor clinical condition demonstrated no proteolytic activity. Twenty-three cystic fibrosis strains of *P. aeruginosa* examined demonstrated reduced bacteremic virulence when compared with a virulent burn strain with a 50% lethal dose (LD_{50}) of 1.5×10^1 CFU in an invasive burned mouse model. Ninety-two percent of the strains tested were avirulent at doses of 10^3 to 10^5 CFU. The LD_{50}s were determined for 10 selected strains which exhibited specific important morphological and physiological deficiencies. Five of the strains tested gave LD_{50}s $> 10^6$ CFU. Reduced virulence of these strains was associated with loss of two or more physiological characteristics associated with virulence. The cystic fibrosis strains of *P. aeruginosa* which morphologically and physiologically resembled the virulent burn strain were the most virulent (LD_{50}s of 10^2 to 10^4). Results suggest that some degree of virulence is associated only with classic strains prevalent in early infections. The data suggest that a selection transition occurs in the lungs of patients with cystic fibrosis that favors *P. aeruginosa* avirulence. The avirulent state may be caused by alterations in the cell envelope, including associated factors such as motility and chemotaxis and protease production.

A principal cause of death in patients with cystic fibrosis (CF) is respiratory failure due to a complex interaction between the host and *Pseudomonas aeruginosa* (6, 10, 14, 17). Recent research suggests that those strains associated with chronic infection differ from those associated with acute infection (8, 19, 27). Several physiological characteristics associated with invasiveness, such as chemotaxis and motility, appear to be altered in the CF strains (16). Results of studies with animal models, using *P. aeruginosa* isogenic mutants, indicate that the lack of these characteristics is associated with reduced invasiveness (18; D. Drake, G. Shaw, and T. C. Montie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B 155, p. 43).

In this study, CF strains of *P. aeruginosa* from patients in good and poor clinical condition were characterized regarding O antigenicity and proteolytic activity. Representative strains with various morphological and physiological traits were then tested for invasiveness using the burned mouse model for acute infection and lethality (26). This measure of virulence can be distinguished from models of animals with CF, in which the degree of localized tissue damage is assessed (28). The results of this research suggest that there is a progressive host selection in the lungs of patients with CF against certain bacterial properties normally associated with bacteremic virulence.

MATERIALS AND METHODS

Bacteria and culture media. All *P. aeruginosa* CF strains listed in Table 1 were isolated from sputum and generously supplied by M. J. Thomassen (Case Western Reserve University). *P. aeruginosa* M-2 was originally isolated from the small intestine of a CF-1 mouse. Strain M-2 was obtained from I. A. Holder (Shriner's Burns Institute).

Maintenance of bacterial cultures in Luria broth and standard growth medium has been described previously (16).

Slide agglutination assay of O antigen. Bacteria were grown for 24 to 30 h at 37°C on tryptone broth agar. Three loopfuls of bacteria were suspended in 2 ml of sterile saline (0.85% [wt/vol]). The bacterial suspension (40 μl) was placed on a glass microscope slide. A 40-μl drop of antiserum (American Scientific; Difco Laboratories, Detroit, Mich.) diluted 10-fold in saline was added to the cell suspension. The slide was rocked back and forth and observed for 2 min. A positive reaction was visible agglutination occurring within the 2-min incubation period (2). Strains were designated as polyagglutinating if they agglutinated with 2 or more of the 17 antisera. The strains which did not agglutinate in any of the 17 antisera were designated nontypable.

Proteolytic activity. Proteolytic activity was assayed based on a modified method of Sokol et al. (25) with equal volumes of 3.0% skim milk and Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar. Cells grown for 15 h on tryptone agar were streaked in single lines on the plates. The zone of hydrolysis around bacterial growth was measured at 24 h.

Laboratory animals. The first set of virulence tests was performed with female Ha/ICR mice (weight, 22 to 24 g; Cumberland View Farms, Clinton, Tenn.). For 50% lethal dose (LD_{50}) determinations, female CF-1 mice (weight, 19 to 21 g) were used (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The mice were used within 2 weeks after their arrival.

Experimental burn procedure. A modification of the burned mouse model of Stieritz and Holder (26) was used to study the virulence of CF strains of *P. aeruginosa*. Instead

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TABLE 1. O antigen serotypes of *P. aeruginosa* strains from patients with CF

Strain	Clinical condition[a]	Colonial morphology[b]	O serotype[c]
86f	G	R	10
415gg	P	R	11
572b	G	R	NT
414nn	P	R	NT
414ii	P	R	NT
402c	P	R	NT
96e	P	R	PA
409g	P	R	PA
66g	P	R	PA
412oo	G	R	PA
320f	G	R	PA
412a	G	M	PA
35c	G	C	6
435c	G	C	PA
903a	G	C	4
902c	G	C	PA
144b	P	C	6
776e	P	C	PA
676e	P	C	PA
541a	P	C	NT
$437e_2$	G	R	ND
412oo	G	R	ND
572d	M	C	ND
448hh	P	R	ND
541a	P	C	ND
M-2[d]		C	5

[a] Clinical evaluation of patients is based on a modification of the scoring system of Schwachman et al. (23) in which a maximal score of 25 points is awarded for each of four categories: chest roentgenogram, general activity, physical activity, and nutrition. Patients in consultation with their physicians are then classified into three categories: 1, good (G), total score of 75 to 100; 2, moderate (M), total score of 60 to 74; 3, poor (P), total score of less than 60.

[b] R, rough; M, mucoid; C, classic.

[c] NT, nontypable, no agglutination in any typing sera; PA, polyagglutinable, agglutinated by two or more typing sera; ND, no data.

[d] An invasive burn strain.

of an asbestos shield a white Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) board with a 35-by-25-mm window was used to circumscribe the ethanol burn area. The mice were anaesthetized before they were burned for 13 s. The actual number of bacteria per inoculum was determined by using plate counts on Trypticase soy agar. A control group received 0.1 ml of M-2 at 1×10^2 CFU per 0.1 ml (LD_{50}, 1.5×10^1 for HA/ICR mice and 5×10^0 for CF-1 mice) injected subcutaneously at the burn site. The number of deaths per group of mice was recorded for 7 days.

RESULTS

Twenty CF strains of *P. aeruginosa* were serotyped with viable cells in a slide agglutination assay (Table 1). Only two of the rough strains (86f and 415gg) were typable, a characteristic associated with intact lipopolysaccharide (LPS) (8). Of the four rough strains which were nontypable, only one (572b) was from a patient in good clinical condition (G strains). Of the rough strains, 45% polyagglutinated with two or more antisera. One-third of the classic strains tested were typable. The majority (55%) of the classical strains were polyagglutinable. However, in contrast to rough strains, only one classic strain (541a) was nontypable. Of the 20 strains tested, 75% were either polyagglutinable or nontypable. Regardless of the clinical condition of the patient or colonial morphology, some type of LPS alterations were present in the majority of strains.

Thirty-two strains were screened for proteolytic activity in skim milk plates, an assay which detects both protease and elastase. Results in Table 2 show that 12 strains demonstrated proteolytic activity comparable to that of M-2. Of these 12 strains, 9 were G strains. In sharp contrast, 13 strains exhibited no proteolytic activity. Of these nonproteolytic strains, 70% were from patients in poor clinical condition (P strains).

TABLE 2. Proteolytic activity of CF strains of *P. aeruginosa*

Strain[a]	No. of strains/total with the following zones of clearing[b]		
	+	±	−
Good	9/32	3/32	4/32
Poor	3/32	4/32	9/32

[a] Each strain was tested at least three times. For each assay CF strains were compared with proteolytic burn strain M-2 (zone of clearing, ≥7 mm).

[b] Zone of clearing (radius) on skim milk plates was measured at 24 h: +, ≥7 mm; ±, ≤3 mm; −, 0 mm.

P. aeruginosa is avirulent in normal mice, but it is extremely lethal in those that receive thermal trauma. In the burned mouse model developed by Stieritz and Holder (26) the LD_{50} for M-2 is 1.5×10^1 CFU following subcutaneous inoculation into the burn site of HA/ICR mice. A comparison of relative virulence revealed significant differences between M-2 and CF strains of *P. aeruginosa* (Table 3). Injection of 310 CFU of M-2 caused 100% mortality in HA/ICR mice by day 3. Of the 23 CF strains examined, only strain 35c exhibited virulence approaching that of M-2 (3.2×10^4 CFU of 35c caused 80% mortality). Injections ranging from 3.5×10^3 CFU to 6.1×10^5 CFU caused 0 to 20% mortality in 97% of the strains tested.

A series of varied morphological types of CF sputum strains were then examined for virulence in the burned

TABLE 3. Comparison of lethality of CF strains of *P. aeruginosa* in the burned mouse model (HA/ICR mice)

Strain	Dose (CFU)[a]	% Lethality[b]
M-2 (control)	3.1×10^2	100
$437e_2$	3.9×10^5	0
572b	4.1×10^4	20
86f	6.1×10^5	0
412oo	5.5×10^3	0
320f	3.5×10^3	20
435c	1.5×10^4	0
903a	4.6×10^3	20
35c	3.2×10^4	80
902c	5.1×10^4	0
412a	8.2×10^3	40
572d	5.6×10^4	0
402c	2.7×10^4	0
409g	3.8×10^4	0
96e	5.3×10^4	0
66g	6.2×10^4	0
415gg	7.2×10^3	0
414ii	3.8×10^4	20
414nn	4.7×10^4	20
448hh	9.3×10^3	0
541a	3.7×10^4	0
144b	3.8×10^5	20
776e	6.7×10^4	0
676e	4.6×10^4	0

[a] Subcutaneous injection of bacteria (in 0.1 ml of buffer) into the burn site.

[b] Lethality of a group of five mice.

TABLE 4. Comparison of lethality of *P. aeruginosa* strains in the burned mouse model (CF-1 mice)

Strain[a]	Colonial morphology[b]	O antigen type[c]	Proteolytic activity[d]	% Motility[e]	% Arginine chemotaxis[e]	LD_{50}[f]
M-2	C	5	+	100	100	5×10^0
35c (G)	C	6	+	45	80	8.4×12^2
903a (G)	C	4	+	106	57	1×10^4
412a (G)	M	PA	+	108	55	3.6×10^4
572b (G)	R	NT	+	108	82	7.1×10^6
320f (G)	R	PA	−	67	31	1.5×10^6
86f (G)	R	10	±	146	96	$>4.0 \times 10^6$
409g (P)	R	NT	−	70	63	$>6.8 \times 10^6$
144b (P)	C	6	−	58	14	$>6.2 \times 10^6$
402c (P)	R	NT	−	46	15	$>6.5 \times 10^6$
776e (P)	C	PA	+	108	37	$>2.4 \times 10^6$

[a] All strains possessed flagella as determined by electron microscopy.
[b] C, classic; M, mucoid; R, rough.
[c] PA, polyagglutinable; NT, nontypable.
[d] Zone of clearing in skim milk plates measured at 24 h (Table 2).
[e] Compared with that of M-2 (100%) by a capillary assay.
[f] Lethality LD_{50}, estimated from four different doses of groups of eight mice. Strains 86f, 409g, 144b, 402c, and 776e demonstrated 0% mortality at 10^6 CFU.

mouse model with CF-1 mice (Table 4). Morphological types tested were smooth and rough strains from patients in good and poor clinical condition. One mucoid strain was also tested. Of the 10 CF strains examined, strains 35c and 903a resembled strain M-2, which had classic morphology and was proteolytic and O antigen typable. Strain 35c was the most virulent of the 10 strains with an LD_{50} of 8×10^2 CFU. Strains 412a and 903a were intermediate in virulence (LD_{50}, 10^4 CFU). Avirulence of the other strains appeared to be associated with the loss of one or more physiological characteristics reported to be associated with virulence. An LD_{50} of greater than 10^6 resulted when two or more virulence factors were absent. For example, strains such as 86f, 409g, 144b, 402c, and 776e had no deaths at 10^6 CFU.

DISCUSSION

In contrast to the 95% of strains from clinical strains other than CF which can be allocated to a specific serotype (2, 3, 19, 21, 22), 75% of the CF strains tested in our investigation were either polyagglutinable or nontypable. Only 2 of the 11 strains tested that had a rough colonial morphology were typable. In contrast to the rough strains, three of the eight classic strains were typable, while only one was nontypable. However, as in the case of rough strains, it was found that half of the classic strains were polyagglutinable. These results are consistent with the recent results of Hancock et al. (8) and Penketh et al. (19), who noted multiple agglutination with O antigen antisera in 50% of the strains tested and a low frequency (32%) of typable strains. It is not known whether strains in this study that were nontypable by agglutination possessed altered O antigen or lacked O antigen entirely. As might be expected, the majority of nontypable strains were of rough colonial morphology. It is important to emphasize that these strains were isolated from patients in poor clinical condition (Table 1). The observation that half of both classic and rough strains were polyagglutinable strongly suggests that the cell wall of CF strains has been modified in a manner not characteristic of *P. aeruginosa* strains associated with acute infections. This unique change in *P. aeruginosa* LPS properties and other virulence factors (16) is separate from the consideration of mucoidy (8), an important phenotypic characteristic of CF strains.

Our serotyping data are consistent with the results of Thomassen and Demko (28), who reported a change of sensitivity of bacteria in serum during the progress of infection associated with patient clinical condition. CF strains that were resistant to autologous serum but sensitive to noncystic serum were primarily from patients in poor clinical condition (27). The latter characteristic of serum sensitivity, which appears to be associated with altered LPS, may explain in part why patients with CF rarely suffer from bacteremia. The containment of *P. aeruginosa* in the lungs of patients with CF can be contrasted to *Pseudomonas pneumonia* infection, which results in an extremely high incidence of bacteremia and mortality (20). In patients with CF, on the other hand, the humoral immune response is generally elevated, and the invading organisms remains localized in the lungs (7).

Two major proteases are associated with *P. aeruginosa* virulence: alkaline protease and elastase (4, 29, 30). Hastie et al. (9) have found that the majority of clinical strains of CF possess proteolytic activity. We tentatively concluded that the proteolytic activity of CF strains is primarily characteristic of the initial, invasive organism. Of the CF strains from patients in good clinical condition, 52% were as proteolytic as strain M-2. Of all strains from patients in good clinical condition, 72% demonstrated proteolytic activity. In sharp contrast, only 20% of CF strains from patients in poor clinical condition were as proteolytic as strain M-2. More striking is the observation that 60% of the strains from patients in poor clinical condition demonstrated no proteolytic activity. Jagger et al. (11) recently have reported somewhat similar results in that more chronically infected patients had more weakly proteolytic strains than patients that were newly colonized. The absence of this important virulence factor in such a large number of strains isolated from chronically infected patients has not been previously reported. It is, however, a logical step in the establishment of persistent infection. Proteases have tissue damaging activity and can degrade anatomical barriers to facilitate the spread of infection (1, 13, 24). The absence of protease activity in patients with chronic infections may reflect the fact that the spreading factor is required less once the organism is established in the lung. Recently, Suter et al. (27) found evidence indicating that the elastase found in bronchial secretions from patients with CF originates from neutrophils rather than from *Pseudomonas* spp. These results are consistent with our findings.

Since many of the CF strains possess phenotypic alterations not manifested in typically invasive isolates, we

suspect that they also may be less virulent when tested in an animal infection model. Results of several studies support this line of reasoning. In a study by Lindberg et al. (15) it was found that *Escherichia coli* strains in patients with asymptomatic bacteriuria had altered cell walls compared with those in patients with symptomatic infections. They hypothesized that the cell wall alteration could reduce virulence. Studies by Montie et al. (18) and Woods et al. (29) with isogenic mutants of *P. aeruginosa* used to test for virulence showed reductions in virulence when a single virulence factor was absent. We examined the relative virulence of the CF strains compared with the virulent burn strain in the burn mouse model. In a preliminary screening of 23 strains, only strain 35c demonstrated virulence comparable to that of M-2. Once the strains were further characterized (16) we selected a series of 10 various morphological types of test for virulence in the burned mouse model. The results of the second set of animal studies were consistent with those of the preliminary screening. Only strain 35c had an LD_{50} approximating that of the virulent burn strain, while two G strains were intermediate in virulence. Avirulence appeared to be associated with loss of one or more physiological characteristics associated with virulence.

Preliminary screening for the presence of plasmids by the method of Kado and Liu (12) indicated that all strains, including M-2, possessed no extrachromosomal material (data not shown). Thus, it is unlikely that a plasmid-mediated virulence factor could account for differences in the virulence observed in this study.

From this investigation the following hypothesis can be presented with respect to the establishment of a chronic *P. aeruginosa* infection in the lungs of patients with CF. The initial invasive CF strain would appear to be similar to strain M-2, the invasive burn strain, in that the former possesses a number of recognized virulence characteristics. As the infection is established in the lungs, host selective pressures, possibly resulting from elevated cation levels (6) and antibiotic pressures, for example, result in the emergence of a different phenotype with rough colonial morphology. First observed in such strains is altered motility and chemotaxis often accompanied by a reduction in proteolytic activity and altered LPS. These physiological alterations are associated with a loss of invasive virulence. Once chronic infection is established in the lungs, we observed that an unusually high number of these strains lack flagella, are protease negative, nonmotile, and nonchemotactic and have altered LPS as reflected in the high frequency of polyagglutinating and nontypable strains. It is notable that these are not transient changes but are apparently genetically stable physiological characteristics (16). It would appear that progressive selective pressure in the chronically infected lungs of patients with CF favors emergence of an avirulent phenotype with an altered cell envelope (5, 8).

ACKNOWLEDGMENTS

We are indebted to M. J. Thomassen (Case Western Reserve University) for supplying the strains and the patient data used in this study. Her critical reading of the manuscript is greatly appreciated. We also acknowledge David Drake for invaluable assistance with the burned mouse model and Malcolm Shields for the plasmid screening.

This work was supported by U.S. Army contracts DAMD 17-82-C-2202 and DAMD 17-85-C-5021.

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