Serotypes and Antibiotic Susceptibilities of *Pseudomonas aeruginosa* Isolates from Single Sputa of Cystic Fibrosis Patients

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A phenotypic characterization of *Pseudomonas aeruginosa* from single sputum samples of 21 typical cystic fibrosis patients indicated a high frequency of heterogeneity among isolates on the basis of differences in antibiotic resistance, colony morphology, pigmentation, and serotype. Two or more isolates with different but stable susceptibilities to carbenicillin, gentamycin, streptomycin, tetracycline, chloramphenicol, and sulfamethoxazole plus trimethoprim were detected in 38% of the sputa. Differences generally were independent of the mucoid state of the strain. O-antigen group determination with the Difco typing set showed that two or more serologically distinct strains were present in 10/21 sputum specimens. Nonmucoid derivatives of mucoid isolates almost always retained both the antibiotic susceptibilities and serotype of their parent strain. These data suggest that cystic fibrosis patients may be cocolonized/coinfected by different strains of *P. aeruginosa* more frequently than generally believed. Alternatively, phenotypically distinct strains from a single patient might arise as phenotypic dissociants from a single infecting strain. Because of the frequency and multiplicity of phenotypically distinct *P. aeruginosa* isolates which we obtained from our cystic fibrosis patients, it is important to select multiple isolates from sputum cultures for antimicrobial susceptibility testing so as to assess adequately the susceptibility of this organism to antibiotic therapy in cystic fibrosis. We recommend that several colonies of each distinguishable colony type of *P. aeruginosa* be pooled for the antibiogram.

Pseudomonas aeruginosa and *Staphylococcus aureus* are the two pathogenic bacterial species most frequently detected in the respiratory tract of patients with cystic fibrosis (CF) (9). The successful control of *S. aureus* by antibiotic therapy has left *P. aeruginosa* as the major microbial threat to life in these patients. Details of the relationships between colonization/infection by *P. aeruginosa* and the pathophysiology of CF have remained obscure. Although *P. aeruginosa* is known to be an opportunistic pathogen which infects the debilitated patient, it is seldom isolated from pediatric patients with chronic respiratory disease other than CF (although there are isolated reports to the contrary [8]). An exact understanding of why patients with CF are especially susceptible to pulmonary colonization/infection by this species is not now available. However, recent evidence suggests that a humorally acquired defect in alveolar macrophage phagocytic activity against *P. aeruginosa* may play an important role. (4;

M. J. Thomassen, B. Boxerbaum, C. Demko, P. Kuchenbrod, R. E. Wood, and B. Tandler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B29, p. 18).

For some time it has been known that *P. aeruginosa* strains isolated from CF sputum cultures are frequently mucoid and that both mucoid and nonmucoid strains often cocolonize cystic individuals (2, 7, 9). Generally, it has been assumed that CF patients are colonized by only a single *P. aeruginosa* strain, in part because most serotyping, phage, and pyocin typing studies have indicated only a single type for both mucoid and nonmucoid isolates (14, 15). Mucoid strains are suggested to be derived from nonmucoid ones (7, 10). In contrast, two reports from different laboratories suggest that cocolonization of cystics by two or more *P. aeruginosa* strains may be relatively frequent (2, 5).

To examine this situation in our patient population and its potential significance with regard to antibiotic therapy, we obtained multiple iso-

lates of *P. aeruginosa* from single sputum samples and examined their morphology, antibiotic susceptibility spectrum, and serotype (O-antigen group). The results that we report here suggest (i) that CF patients frequently may be cocolonized/coinfected with distinctly different strains of *P. aeruginosa*; (ii) that strains with a variety of different antibiotic sensitivities may coexist within the same cystic individual; and (iii) that although mucoidy or nonmucoidy of a strain may be associated with differences in sensitivity to certain antibiotics, this characteristic in itself does not account for all of the heterogeneity in antibiotic resistances that we have observed in *P. aeruginosa* isolates from our patients.

MATERIALS AND METHODS

Isolation of microorganisms. Sputum specimens were collected in sterile containers from CF outpatients (age range 4 to 20 years) who had productive coughing or who became productive during respiratory therapy. These specimens were plated within 2 h. In most cases these specimens were solubilized in Sputolysin (6.5 mM dithiothreitol in 0.05 M phosphate buffer, pH 7.0, supplied by Calbiochem) by mixing an equal volume of sputum and 10% Sputolysin. After mixing on an orbital mixer and centrifugation ($10,000 \times g$ for 15 min), the specimens were streaked on blood agar, chocolate agar, eosin-methylene blue agar, and pseudomonas isolation agar (Difco) or for quantitative cultures were diluted and pipetted onto plates with an Eppendorf pipetter (Scientific Products). Plates were inverted and incubated at 37°C.

Identification of *P. aeruginosa*. Because several species of *Pseudomonas* have been isolated on occasion from CF patients and mistaken identification can explain unexpected antibiotic sensitivity patterns and ambiguities in serotyping, we have made a rigorous attempt to establish that each of our isolates was indeed *P. aeruginosa*. To insure that we were working with pure cultures, strains were serially restreaked at least three times after isolation. Strains isolated from other media were examined for growth and pigmentation on pseudomonas isolation agar. All isolates were confirmed as *P. aeruginosa* by their biochemical reactions in the API 20E System (Analytab Products), by their positive response on acetamide agar (indicator color change) (Cal Labs), by their positive oxidase test (Cepti Seal oxidase reagent, Marion Scientific Corp.), by their motility in hanging drop preparation, and by growth at 42°C.

Antibiotic susceptibility testing. The antibiotic susceptibility of 2 to 10 separate primary isolates of *P. aeruginosa* from each sputum specimen was determined by the Kirby-Bauer method (1) for rapidly growing pathogens slightly modified to include recommendations by the National Committee on Clinical Laboratory Standards. Testing of eight antibiotics with a variety of mechanisms of action was performed on plates of Mueller-Hinton agar supplied by Oklahoma Biologics. Different lots of Mueller-Hinton agar were assayed with standard cultures to assure uniformity. Antibiotic disks, carbenicillin, chloramphenicol,

sulfamethoxazole plus trimethoprim (SXT), tetracycline, colistin, streptomycin, gentamycin, and tobramycin were supplied by Pfizer Diagnostics. Minimal inhibitory concentrations were determined in microtiter plates prepared in our laboratory (Cooke) to which we added Mueller-Hinton broth (0.25 ml) and an equal volume of fresh culture. Minimal inhibitory concentrations of SXT were determined on Mueller-Hinton agar.

Serotype determination. *P. aeruginosa* serotypes (O antigen) for selected isolates (ones derived from single sputum specimens and which were phenotypically distinct from coisolates) from 10 patients were established by *P. aeruginosa* antisera (Difco) with *P. aeruginosa* antigens (Difco) as positive controls. The slide agglutination assay was performed exactly as described on the package enclosure with a freshly prepared, autoclaved (30 min at 121°C), and washed bacterial suspension as the test antigen. Bacteria were grown on nutrient agar. Each strain was tested for autoagglutination in a normal (nonimmunized) control rabbit serum.

RESULTS

P. aeruginosa was isolated from 21 of 25 (84%) sputum specimens from CF patients seen at the Cystic Fibrosis Center of Oklahoma Children's Memorial Hospital during a 3-month period. *P. aeruginosa* was the microorganism most commonly isolated; although mixed infection with *S. aureus* occurred with an appreciable frequency (20%), other microorganisms occasionally detected in CF sputa were infrequent in our specimens (Table 1). Sputum from only one patient contained normal flora. Cocolonization/coinfection by mucoid and nonmucoid strains of *P. aeruginosa* was detected in 56% of the sputa (Table 1). From these observations, the bacterial flora of our patient population appeared to be quite typical of that generally observed for patients with CF (9).

Quantitation of *P. aeruginosa* (summarized in Table 1) showed that the concentration of viable cells ranged from 2×10^3 to 2×10^8 /ml of solubilized sputum. The use of pseudomonas isolation agar facilitated the detection of *P. aeruginosa* colonies when other pathogens and normal flora were present in high numbers (as in the patient with *P. aeruginosa* and *S. aureus* and *Haemophilus influenzae*) but *P. aeruginosa* concentration was $\leq 10^3$ cells per ml of sputum. Of 21 patients, 14 had *P. aeruginosa* concentrations between 3×10^5 and 5×10^7 cells per ml. There was no obvious correlation between the presence of significant concentrations of *S. aureus* or *H. influenzae* and increased or decreased concentrations of *P. aeruginosa*.

When the antibiotic susceptibilities of different *P. aeruginosa* isolates from a single sputum specimen were compared, frequent differences

TABLE 1. Microorganisms detected in sputum from 25 patients with CF

Organisms	<i>Pseudomonas</i> colony type	Quantitation ^a		No. of patients
		<i>Pseudomonas</i>	Others	
<i>P. aeruginosa</i> only	Nonmucoid only	1×10^6 – 2×10^7		2
	Mucoid only	4×10^4 – 5×10^7		5
	Nonmucoid + mucoid	2×10^3 – 2×10^8		7
<i>P. aeruginosa</i> + <i>S. aureus</i>	Nonmucoid only	2×10^3 – 3×10^4	4×10^5 – 4×10^7	2
	Mucoid only			0
	Nonmucoid + mucoid	5×10^3 – 5×10^5	6×10^4 – 4×10^7	3
<i>P. aeruginosa</i> + <i>S. aureus</i> + <i>H. influenzae</i>	Nonmucoid only	2×10^3	4×10^7 ; 4×10^7	1
<i>P. aeruginosa</i> + <i>H. influenzae</i>	Nonmucoid only	3×10^7	6×10^6	1
<i>S. aureus</i> only			4×10^6 – 5×10^7	2
<i>H. influenzae</i> only				0
β -Hemolytic streptococci only			1×10^6	1
Normal flora only				1

^a Quantitation refers to the number of colony-forming units per milliliter of solubilized sputum expressed as a range. Values under *Pseudomonas* heading are for total *Pseudomonas* count; those listed under Others are for the respective, specific other microorganism(s).

in the levels of sensitivity to six of the eight antibiotics were observed among the isolates. Colistin and tobramycin were exceptional in that all isolates were uniformly sensitive. As examples of the heterogeneity of resistance levels among strains isolated from a single sputum sample, 21% of the sputa yielded strains with demonstrable differences in susceptibility to gentamycin (sensitive, intermediate, and resistant types). Fifty percent of the sputa yielded strains with different levels of resistance to SXT. When the antibiotics are viewed singly, approximately 20% of the sputa contained *P. aeruginosa* strains heterogeneous in their susceptibility to five of the six antibiotics. The SXT mixture was the antibiotic for which the isolates were most frequently heterogeneous (50%).

Examples of susceptibility differences for a few representative strains are indicated in Table 2. The susceptibilities of these strains and arbitrarily selected other isolates have been independently determined several times to assure their reliability. Not all of this work was performed with the same lot of Mueller-Hinton agar plates. Variability between plates as an explanation of these results is obviated by examination of two strains from a sputum specimen on the same plate. As shown in Fig. 1, the differences in antibiotic susceptibility are maintained when this is done. On the basis of the combinations of susceptibility to the whole group of antibiotics, 8 of 21 sputum specimens (38%) had two or more phenotypically distinct strains.

The strains described in Table 2 were chosen for inclusion because they suggest the spectrum of heterogeneity for a variety of phenotypic attributes which characterizes *P. aeruginosa* strains isolated from CF sputum. In addition to differences in antibiotic susceptibility, there were frequent, clear-cut differences in slime production (mucoidy), colony morphology, pigment production, and often O antigen. One inference that might be drawn from Table 2 is that frequently there may be a relationship between mucoidy and antibiotic susceptibility (e.g., the transition from nonmucoid to mucoid, or the converse, might be accompanied by concomitant change in chloramphenicol resistance as suggested by isolates from patient 4). Two different observations make it unlikely that the mucoid state of the isolates accounts for the heterogeneity for drug resistance that we have observed. (i) From one patient, 10 mucoid, morphologically indistinguishable isolates were obtained from a single sputum sample. These were restreaked three times and then tested for their susceptibility to the antibiotic test set. Three different phenotypes were identified with regard to carbenicillin and SXT: Car^R SXT^R, Car^S SXT^S, and Car^I SXT^R. These differences were confirmed by minimal inhibitory concentrations; in addition, two different sensitive levels were found for SXT (4 and 64 μ g/ml). (ii) Generally, there was no consistent relationship between antibiotic susceptibility determined by the Kirby-Bauer method and mucoidy (the latter

TABLE 2. Examples of phenotypic differences among *P. aeruginosa* isolates from single sputum samples of patients with CF

<i>P. aeruginosa</i> phenotype ^a											
Patient no.	Strain	Antibiotic resistance pattern						Pigment production	Colony morphology	Mucoid colony type	O-antigen group
		Car	Chl	Gen	SXT	Str	Tet				
1	ECO-1	S(18)	I(13)	S(16)	R(6)	R(6)	I(17)			+	9
	ECO-2	R(10)	R(6)	S(17)	R(6)	R(6)	R(6)			—	NA
2	TVI-1	S(42)	R(6)	S(23)	R(6)	S(15)	R(13)	Red-brown		+	10
	TVI-2	S(30)	R(6)	R(10)	R(6)	R(6)	R(14)	Red		—	8,3
3	DFR-1	S(32)	I(13)	S(17)	S(22)	R(8)	I(17)	Aqua		+	8
	DFR-2	S(35)	R(6)	R(12)	R(8)	R(6)	R(13)	Green		—	8
4	SDI-1	S(33)	S(22)	S(22)	S(30)	I(13)	R(14)	Red-brown		+++	9
	SDI-2	S(19)	R(6)	S(17)	R(6)	R(6)	R(12)	Green		—	9
5	TMO-1	S(21)	R(6)	S(21)	R(6)	I(12)	I(16)			+	9
	TMO-2	I(15)	R(6)	S(16)	R(6)	R(6)	R(6)			—	4
	TMO-3	R(13)	R(6)	S(15)	R(6)	R(6)	R(6)		Small, rough	—	12

^a Car, Carbenicillin; Chl, chloramphenicol; Gen, gentamycin; SXT, sulfamethoxazole plus trimethoprim; Str, streptomycin; Tet, tetracycline; S, sensitive; R, resistant; I, intermediate sensitivity. The numbers in parentheses reflect zone size in millimeters. When no entry appears under pigment production or colony morphology, these traits were neither remarkable nor recognizably different between isolates. Serotype was determined with the *P. aeruginosa* antisera kit (Difco). NA, Non-agglutinating with the 17 type-specific antibodies. +, Mucoid; —, nonmucoid.

FIG. 1. Antibiotic susceptibility of two different *P. aeruginosa* isolates from a single sputum specimen. The upper half of the plate of Mueller-Hinton agar was spread with one strain, and the lower half was spread with a second strain previously shown to differ from the first in its sensitivity to SXT (middle disk) but not to streptomycin (left disk) or gentamycin (right disk). A marked difference in susceptibility is indicated by the large zone surrounding the SXT disk of the strain on the lower half of the plate as compared with that on the upper half.

scored as discernible mucoid colonies on agar plates by 48 h). For example, both mucoid and nonmucoid colonies (from the same or different sputum specimens) can be either resistant or sensitive to carbenicillin or chloramphenicol. To examine this point in greater detail, we isolated stable, spontaneous nonmucoid segregants from

10 of our primary mucoid isolates and compared their antibiotic susceptibility to that of the parental isolate from which they were derived. Nonmucoid segregants (1 to 10 from each mucoid parent strain) were isolated from plates of pseudomonas isolation agar or eosin-methylene blue agar inoculated with an overnight nutrient broth culture of a mucoid isolate grown at 37°C. These segregants, representing 1 to 90% of the colony-forming units, were nonmucoid after 48 h of incubation when viewed with a dissecting microscope. After purification by streaking, the nonmucoid derivatives were tested for their susceptibilities. The antibiotic susceptibility pattern of these nonmucoid derivatives was most commonly very similar to or indistinguishable from that of their parental mucoid strain. However, these nonmucoid derivatives were readily distinguishable from the nonmucoid cultures originally coisolated with mucoid strains from single sputa. Examples of this finding are shown in Table 3.

These data taken together suggest that *P. aeruginosa* isolates from single sputum samples of our patient population are frequently heterogeneous with regard to their susceptibility to antimicrobial agents. The sensitivity of an isolate appears to be generally a stable characteristic which is not directly related to its mucoid state. An implication of these findings is that certain of the strains that coexist in the CF patient may be of independent origin. If this view is correct, then serological differences

TABLE 3. Comparison of antibiotic susceptibility of mucoid *P. aeruginosa* isolates, their nonmucoid derivatives, and nonmucoid isolates from the same sputum sample

Patient	Strain	O-antigen group	Mucoid colony type	Antibiotic susceptibility						
				Car	Chl	Gen	SXT	Str	Tet	Tob
1	CHI-1	9	+	S(38)	R(6)	S(22)	S(28)	R(11)	R(11)	S(28)
	CHI-1D	9	—	S(31)	R(6)	S(20)	S(28)	R(6)	R(11)	S(26)
	CHI-2	3	—	S(22)	R(6)	I(13)	R(6)	R(6)	R(6)	S(19)
2	DFR-1	8	+	S(32)	I(13)	S(17)	S(22)	R(11)	I(17)	S(23)
	DFR-1D	8	—	S(30)	I(13)	S(18)	S(21)	R(11)	I(17)	S(22)
	DFR-2	8	—	S(28)	R(6)	R(12)	R(8)	R(11)	R(10)	S(19)
3	MJE-1	9	+	S(35)	S(18)	S(28)	S(24)	S(18)	S(32)	S(23)
	MJE-1D	A	—	S(28)	S(17)	S(27)	S(18)	S(17)	S(24)	S(22)
	MJE-2	8	—	S(27)	R(10)	S(15)	R(6)	R(8)	R(10)	S(18)

" Susceptibility was determined by the Kirby-Bauer method. Abbreviations used are the same as in Table 2. A, Auto-agglutinating. The nonmucoid derivatives of the mucoid strains (designated by D) were stable, spontaneous segregants obtained from overnight cultures grown in nutrient broth at 37°C, and then purified by streaking. Numbers in parentheses are zone diameters in millimeters. +, Mucoid; —, nonmucoid.

among isolates from a single sputum specimen might occur commonly. To examine this point further, we determined with commercially supplied sera the O-antigen group of *P. aeruginosa* isolates from 21 sputum samples. The results presented in Table 2 are representative of the occurrence of serologically distinct isolates which we detected in single sputum specimens. Such differences were common; they were observed in 10 of 21 sputa. Although mucoid strains were sometimes serologically distinct from nonmucoid ones (e.g., ECO-1 and ECO-2 or TVI-1 and TVI-2 in Table 2), there was no fixed relationship between mucoid state and O-antigen group (e.g., compare in Table 2 SDI-1 and SDI-2 or TMO-2 and TMO-3). Stable nonmucoid segregants derived from 10 mucoid strains retained the serotype of their parental strains. Representative examples are shown in Table 3. The CHI isolates of Table 3 are typical in that mucoid and nonmucoid coisolates from a single sputum sample have different O-antigen types (CHI-1 versus CHI-2), whereas the nonmucoid derivative strain (CHI-1D) is antigenically similar to the mucoid strain. A single exception among 20 nonmucoid segregants was autoagglutinating (MJE-1D in Table 3).

DISCUSSION

Chronic and recurrent respiratory infections caused by *P. aeruginosa* and other opportunistic pathogens are characteristic of CF. Despite therapeutic progress (9, 12), pulmonary disease continues to be the most frequent cause of death in CF (6). The specificity of the site of infection and the rarity of systemic spread of infection in CF are inconsistent with an intrinsic defect in the immune system, and no such intrinsic defect has been convincingly demonstrated in vitro.

Nonspecific impairment of the pulmonary defenses, for example, impaired mucociliary clearance (11) and/or reduced alveolar macrophage phagocytic activity (4; M. J. Thomassen et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B29, p. 18), may occur as a consequence of the accumulation of bioactive CF factors (3). A partial, progressive impairment in pulmonary defense against infection is consistent with the spectrum of abnormal flora cultured from CF sputum. Organisms with even marginally enhanced resistance to host defenses (e.g., mucoid *P. aeruginosa* [13]) are likely to persist in the lungs of CF patients. Because several different genera of pathogenic bacteria may cocolonize/infect the cystic respiratory tract, most commonly *P. aeruginosa*, *S. aureus*, and *H. influenzae*, perhaps it should not be surprising to find cocolonization/coinfection by more than one strain of *P. aeruginosa*.

Our results are consistent with the view that cocolonization/coinfection by two or more strains of *P. aeruginosa* is common in our CF patient population. These strains may be distinguished phenotypically on the basis of antibiotic susceptibility, colony morphology, slime production, pigment accumulation, and O-antigen group. Most investigators have distinguished only a single serotype or pyocin type among *P. aeruginosa* isolates from single sputum specimens (15, 17). However, two laboratories with patient populations geographically distinct from ours and using different methods also found frequent heterogeneity on the basis of typing studies (2, 5). With the commercial availability of standardized serotyping kits for *P. aeruginosa* with a broader type selection than heretofore available, it should be possible to evaluate whether such heterogeneity is specifically char-

acteristic of CF patients and a common finding. Although the detection of phenotypically distinct (especially serologically distinct) isolates from single sputa is consistent with primary infection/colonization by more than one strain, the possibility also exists that such variants arise in vivo by "dissociation," i.e., continuous segregation of phenotypic variants arising from a single progenitor strain, as described by Zierdt and Schmidt (16). The phenotypes of our limited number of nonmucoid derivatives were not consistent with this latter interpretation but in themselves do not rule out the inpatient origin of phenotypically discrete isolates.

Among our *P. aeruginosa* isolates from single sputa, heterogeneous but reproducible antibiotic susceptibility was encountered frequently for six different antibiotics. Thomassen et al. (M. J. Thomassen, B. Boxerbaum, and C. A. Demko, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C126, p. 56) reported similar heterogeneity, but their strains did not give reproducible levels of susceptibility upon repeated testing. With *P. aeruginosa*, media composition differences can apparently cause changes in the cell envelope (M. A. Kenward, M. R. W. Brown, and J. J. Fryer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, A7, p. 2) or in the amount of slime produced in liquid medium (R. Chan, J. Lam, R. T. Irvin, and J. W. Costerton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, A13, p. 3) and may result in changes in the apparent susceptibility to certain antibiotics. Different susceptibilities among our isolates from a single sputum specimen could not be explained simply on the basis of amount of slime production because nonmucoid derivatives possessed susceptibilities similar to their mucoid parent strains, and all strains were tested and maintained on the same medium.

There is often a difficulty in eradicating *P. aeruginosa* from the respiratory tract of the CF patient by antibiotics to which conventional clinical methods indicate the culture should be sensitive. Unresponsiveness to the appropriate antibiotics may reflect problems in combating pulmonary infection which are inherent to patients with CF. Antibiotic diffusion and availability may be significantly different in the sputum of the patients, and the decreased alveolar macrophage phagocytic activity or other factors may impair the anticipated response to treatment. In addition, susceptibility as determined in vitro may not reflect the in vivo sensitivity of the strain(s). Finally, the decreased effectiveness of antimicrobial agents may arise in part from intrinsic differences in susceptibility among different strains of *P. aeruginosa* coincidentally harbored by an individual at any given time. In view

of the complexity and multiplicity of phenotypically distinct *P. aeruginosa* isolates which we obtained from our CF patients, it appears to be important to select multiple isolates from sputum cultures for antimicrobial susceptibility testing so as to assess adequately the susceptibility of this organism to antibiotic therapy in CF.

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