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Adherence of *Pseudomonas aeruginosa* to Tracheal Epithelium

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Adherence of mucoid and nonmucoid strains of *Pseudomonas aeruginosa* to tracheal epithelium was studied with a perfused-trachea model. The species specificity of adherence was studied by infecting tracheas from hamsters, guinea pigs, or mice. Perfused tracheas from hamsters were infected with strains of *P. aeruginosa* in the presence of various sugars, lectins, cations, or charged polymers. Adherence of mucoid strains of *P. aeruginosa* was greatest for guinea pigs; that for hamsters and mice was approximately the same. Nonmucoid strains did not adhere well to epithelium from any of the species tested. *N*-Acetylglucosamine, galactose, and *N*-acetylneuraminic acid were the best inhibitors of adherence of mucoid strains of *P. aeruginosa*. *Phaseolus vulgaris* agglutinin and *Arachis hypogea* agglutinin enhanced adherence of mucoid strains. Adherence of mucoid strains was also enhanced by the presence of Ca^{2+} in the incubation medium. Poly-L-lysine, poly-L-aspartic acid, and polyglycine inhibited adherence of a mucoid strain by 96, 86, and 52%, respectively. In general, the adherence of nonmucoid strains was not affected. The results indicate that carbohydrates are involved in the interaction of mucoid strains of *P. aeruginosa* with tracheal cells and that divalent cations may enhance this interaction. The lectin data show that lectins can interact with the mucoid organisms and the host and suggest that lectins may play a role in the adhesion process.

Pseudomonas aeruginosa is the major cause of morbidity and mortality in cystic fibrosis (CF) patients, in whom it causes acute, recurrent, and chronic pulmonary infections (6, 7). The organisms isolated from chronic lung infections produce a mucoid exopolysaccharide (MEP) composed of an acetylated polymer of 1,4-linked D-mannuronic and guluronic acids (2). The MEP appears to protect the established organisms from phagocytosis (13) and the activity of pulmonary surfactants (4) and antibiotics (5). The importance of this polysaccharide in colonization has also been demonstrated. The MEP mediates attachment of mucoid strains of *P. aeruginosa* to tracheobronchial mucins (11, 14) and to ciliated cells of the lower respiratory tract (1, 9).

The attachment of *P. aeruginosa* to acid-injured trachea and mucous glycoproteins has been well characterized (11, 12). In previous reports, we demonstrated that mucoid strains of *P. aeruginosa* attached to the normal ciliated epithelium in higher numbers than nonmucoid strains (1, 9), but the mechanism of attachment was not characterized.

We hypothesized that the MEP was an adhesin which interacted with the glycocalyx on the surface of the cilia. Recent evidence using monoclonal antibodies to MEP confirms the role of MEP in adherence (N. R. Baker, and A. Austria, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D102, p. 89). In the present study, the mechanism of attachment of mucoid strains of *P. aeruginosa* was examined by testing various monosaccharides for inhibition of adherence. The effects of cations, lectins, and charged polymers on adherence were also tested. Furthermore, variability in adherence of *P. aeruginosa* strains to tracheal epithelia from several species of animals was investigated.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* 105M and 214NM used in this study were clinical mucoid and nonmucoid isolates obtained from sputa of CF patients at Children's Hospital, Columbus, Ohio. Strain PAO579, obtained from J. Govan (University of Edinburgh, Edinburgh, Scotland), was a mucoid variant of strain PAO381 (5). The mucoid isolates were maintained on pseudomonas isolation agar, and the nonmucoid isolate was maintained on Trypticase soy agar. Stock cultures were stored on Trypticase soy agar slants covered with Trypticase soy broth containing 30% glycerol in liquid nitrogen or at −70°C.

Quantitative perfused-trachea adherence assay. Quantitation of adherence to perfused tracheal epithelium was done as described previously (9). Adult male Syrian golden hamsters were sacrificed by intraperitoneal injection with 0.3 ml of 10% sodium brevitol. Each trachea was excised, cut to 11 mm in length, and placed in the groove in the perfusion chamber. The ends of the trachea were blocked with Plexiglas blocks, and minimal essential medium (MEM) containing 1.5% agarose at 45°C was poured over the trachea. When the agar had solidified, the blocks were removed, any agar trapped in the open ends of the trachea was removed carefully, and the trachea was perfused with MEM containing 1.2% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (MEM-HEPES) (pH 7.4).

Overnight (18- to 20-h) cultures of the *P. aeruginosa* isolates were obtained by inoculating 10 ml of the dialysate of Trypticase soy broth with an isolated colony from an agar plate and incubating the culture at 37°C with shaking at 180 rpm. The concentration of the culture was estimated by comparing the optical density at 540 nm to a standard curve and was adjusted to 10^7 CFU/ml in MEM-HEPES without washing. The actual concentration of inoculum was confirmed by standard plate count on Trypticase soy agar. A total volume of 4 ml of inoculum was added to each chamber, and the explants were incubated at 37°C in a 5% Co_2–air atmosphere for 2 h. At the end of the incubation period, the bacterial suspension was removed and the explants were washed three times with 4 ml of MEM-HEPES each time to remove nonadherent organisms. The posterior and anterior cut ends of the trachea were removed, and a 4-mm section of the trachea was homogenized in 1 ml of sterile double-distilled water in a Ten Broeck tissue grinder. An additional 2 ml of double-distilled water was added to

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rinse each grinder, and the homogenate was diluted and plated on Trypticase soy agar plates for quantitation.

Species specificity of adherence of mucoid and nonmucoid strains of *P. aeruginosa*. The quantitative perfused-trachea adherence assay was done with tracheas excised from adult male hamsters (Syrian), guinea pigs (Harley), or mice (BALB/c). The same perfusion chambers were used for the hamster and guinea pig assays, but a smaller chamber was used for assays with mice. The length of the section of trachea used for quantitation was kept constant at 4 mm. The surface area of the epithelium of each explant was calculated, and adherence was expressed as CFU per square millimeter of tracheal epithelium.

Reagents. The following reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.): D-(+)-galactose (Gal), D-(+)-mannose, L-(−)-fucose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid, bovine submaxillary mucin, *Triticum vulgaris* (wheat germ) agglutinin, *Arachis hypogea* (peanut) agglutinin, *Tetragonolobus purpureas* (lotus) aggulutinin, *Phaseolus vulgaris* agglutinin, *Lens culinaris* agglutinin, polyglycine (type 1, 6,000 molecular weight), poly-L-aspartic acid (sodium salt, 15,000 molecular weight), and poly-L-lysine (hydrobromide, 14,000 molecular weight). Each reagent was dissolved in double-distilled water or MEM-HEPES (pH 7.4) and diluted to the appropriate concentration in MEM-HEPES.

Effects of sugars, mucin, lectins, divalent, cations, or charged polymers on adherence. Each reagent was mixed to the chosen final concentration with the bacterial inoculum immediately prior to inoculation of the perfused explant. The divalent cations tested were solutions of $CaCl_2$ and $MgSO_4$. Controls were infected with bacteria in MEM-HEPES only, and all explants were incubated and processed for quantitation of adherent organisms as described previously.

In an attempt to determine whether the inhibiting sugars were binding to the bacterial adhesins, bacteria were incubated with the appropriate sugar at 1 mg/ml for 15 min at 25°C. The bacteria were then washed three times in 5 ml of MEM-HEPES each time by centrifugation at 12,000 × *g* for 10 min and suspended at 10^7 CFU/ml for use as an inoculum. Control bacteria were incubated in MEM-HEPES and washed as described above. As an additional control, the adherence of an unwashed preparation of bacteria was determined.

The dose response for inhibition of adherence of the mucoid strains of *P. aeruginosa* was determined by infecting the perfused tracheas in the presence of various concentrations of inhibitory agents. Gal and GlcNAc were used at 1,000, 100, 10, and 1 μg/ml, whereas mucin was tested at 10, 1, or 0.1 μg/ml.

RESULTS

Species specificity of adherence. Comparison of the numbers of organisms adherent to guinea pig, hamster, or mouse tracheal explants revealed no significant differences (Table 1). The number of mucoid organisms adhering to hamster tracheal epithelium was somewhat less than that observed for the other species, but adherence values higher for mucoid organisms than for nonmucoid organisms were confirmed.

Effects of sugars and mucin on adherence. Adherence of mucoid strains of *P. aeruginosa* to tracheal epithelium was inhibited by all the carbohydrates tested when they were present at 1 mg/ml (Table 2). *N*-Acetylneuraminic acid was inhibitory at 0.2 mg/ml (Table 2). When the concentration of the monosaccharides was reduced to 0.1 mg/ml, only GlcNAc and Gal inhibited adherence (Table 2). Reduction of the concentration of these sugars to 0.01 mg/ml resulted in loss of inhibition of adherence (not shown). Mucin clearly inhibited adherence of the mucoid strains to tracheal epithelium (Table 2). Titration of the mucin revealed that the mucin was inhibitory at concentrations as low as 1 μg/ml. Adherence of the nonmucoid strain was not inhibited by the sugars or mucin, but mannose caused a slight increase in adherence (Table 2).

TABLE 1. Adherence of *P. aeruginosa* strains to guinea pig, hamster, and mouse tracheal epithelium

Animal	No. (10^3) of CFU (±SEM)/mm^2 of trachea for strain[a]:	
	105M	244NM
Guinea pig	16 ± 4.7	0.03 ± 0.02
Hamster	4 ± 0.2	0.06 ± 0.04
Mouse	11 ± 0.3	0.06 ± 0.03

[a] Each value is the average of two experiments.

We attempted to determine if the inhibitors were acting on the bacteria or on the epithelium by preincubating bacteria or explants with the sugar and then washing them to remove free inhibitors. However, washing the bacteria caused a significant reduction in adherence, probably due to the loss of the loose exopolysaccharide. The adherence of treated washed bacteria was not different from the adherence of control washed bacteria.

Effect of lectins on adherence. Two lectins, PHA and *A. hypogea* agglutinin, which belong to the GalNAc-Gal group of lectins, enhanced adherence of the mucoid strain of *P. aeruginosa* (Table 3). Adherence of the nonmucoid strain was not altered by the lectins tested.

Effect of calcium or magnesium on adherence. The effects of calcium and magnesium on adherence were different for the two mucoid strains tested. Adherence of strain 105M was

TABLE 2. Effects of sugars and mucin on adherence of *P. aeruginosa* strains

Treatment	Concn (mg/ml)	No. (10^3) of CFU (±SEM)/4 mm of trachea for strain[a]:	
		105M	244NM
None	0	220 ± 32	3.6 ± 1.2
Mannose	1.0	21 ± 11[b]	8.7 ± 1.4[b]
	0.1	149 ± 6	NT[c]
GlcNAc	1.0	47 ± 15[b]	2.0 ± 0.4
	0.1	26 ± 8[b]	NT
GalNAc	1.0	65 ± 13.5[b]	3.9 ± 0.6
	0.1	141 ± 7.5	NT
Gal	1.0	35 ± 9.5[b]	5.7 ± 1.25
	0.1	41 ± 11[b]	NT
Fucose	1.0	37 ± 6.5[b]	2.9 ± 0.75
	0.1	148 ± 9.5	NT
N-Acetylneuraminic acid	0.2	34 ± 15.5[b]	4.1 ± 0.95
	0.1	217 ± 63	NT
Mucin	0.04	12 ± 2[b]	2.6 ± 0.6
	0.01	13 ± 2[b]	NT

[a] Each value is an average of four experiments.
[b] Significantly different from CFU adhering in the absence of added agents ($P < 0.05$) as determined by the *t* test.
[c] NT, Not tested.

TABLE 3. Effects of lectins on adherence of *P. aeruginosa* strains

Source of agglutinin (100 μg/ml) for treatment	Lectin specificity	No. (10^3) of CFU (±SEM)/4 mm of trachea for strain[a]:	
		105M	244NM
None	0	136 ± 14	1.8 ± 0.27
T. vulgaris	GlcNAc	214 ± 84	2.2 ± 0.31
PHA	Galβ1-4GlcNAcβ1-2Man	759 ± 282[b]	1.8 ± 0.22
T. purpureas	αL-Fucose	340 ± 63	2.1 ± 0.27
A. hypogea	Galβ1-3GalNAc	1660 ± 335[b]	1.8 ± 0.22
L. culinaris	αMan > αGlc, αGlcNAc	408 ± 165	0.9 ± 0.05[b]

[a] Each value is an average of five experiments.
[b] Significantly different from CFU adhering in the absence of added agents ($P < 0.05$) as determined by the *t* test.

enhanced by both cations (Table 4), whereas adherence of strain 579M was enhanced only when the Ca^{2+} concentration was raised to 15 mM. Mg^{2+} did not affect the adherence of strain 579M (Table 4). Adherence of the nonmucoid strain was enhanced by Mg^{2+} but not by Ca^{2+} (Table 4).

Effect of homocharged polymers on adherence. Poly-L-lysine, poly-L-aspartic acid, and polyglycine inhibited adherence of mucoid stains of *P. aeruginosa* by 96, 80, and 52%, respectively (Table 5). None of the polymers inhibited adherence of the nonmucoid strains. The effects of the polymers on viability of the organisms were determined by doing a plate count of the inoculum after the 2-h infection of the explants. No loss of viability of the organisms was observed.

DISCUSSION

The events which lead to colonization of the lungs of CF patients by mucoid strains of *P. aeruginosa* have not been elucidated, but several studies have suggested important steps. Nonmucoid strains typical of early colonization of CF patients appear to attach to upper respiratory tract cells which have been stripped of a layer of fibronectin (16). Fimbriae have been shown to be the adhesins for these organisms (17). The mucoid strains may arise as variants of the nonmucoid strains and colonize the lower respiratory tract. The mucoid variants can bind to tracheobronchial mucins which plug the bronchioles (13, 14) and to cilia of the ciliated epithelium (1, 9). Antibodies to MEP block adherence of mucoid strains of *P. aeruginosa* to mucin (11) and to

TABLE 4. Effect of calcium or magnesium on adherence of *P. aeruginosa* strains to tracheal explants

Cation	Concn (mM)	No. (10^3) of CFU (±SEM)/4 mm of trachea for strain[a]:		
		105M	579M	214NM
None	0	280 ± 65	160 ± 20	3.1 ± 0.15
Ca^{2+}	5	520 ± 55[b]	290 ± 45	3.1 ± 0.10
	10	500 ± 25[b]	320 ± 60	3.9 ± 0.10
	15	1870 ± 210[b]	1430 ± 110[b]	3.0 ± 0.30
Mg^{2+}	5	510 ± 10[b]	210 ± 30	5.9 ± 0.60
	10	510 ± 75[b]	240 ± 32	12.2 ± 0.85[b]
	15	860 ± 240[b]	240 ± 75	14.2 ± 1.0[b]

[a] Average of four experiments.
[b] Significantly different from the control value for that strain ($P < 0.05$) as determined by the *t* test.

TABLE 5. Effects of homocharged polymers on adherence of *P. aeruginosa* strains to tracheal explants

Polymer (100 μg/ml)	No. (10^3) of CFU (±SEM)/4 mm of trachea for strain[a]:	
	105M	244NM
None	138 ± 3	1.5 ± 0.11
Poly-L-lysine	5 ± 0.5[b]	1.8 ± 0.11
Poly-L-aspartic acid	28 ± 2[b]	1.4 ± 0.15
Polyglycine	67 ± 7.5[b]	1.5 ± 0.05

[a] The bacteria were mixed with the polymer before inoculation of the explants. Average of four experiments.
[b] Significantly different from the control value for that group ($P < 0.05$) as determined by the *t* test.

tracheal cells (Baker and Austria, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987), indicating that MEP is an adhesin.

The results of the current study indicate that there is no species specificity when adherence to tissues from several rodents is compared. Adherence of mucoid strains of *P. aeruginosa* to human ciliated tracheal cells has also been reported, indicating a broad host range (3). Adherence of the mucoid and nonmucoid strains was distinct. As we reported previously, the nonmucoid strains did not adhere as well as the mucoid strains. In general, adherence of the nonmucoid strains was not altered by the agents which affected the adherence of mucoid strains. Inhibition of attachment by poly-L-lysine is consistent with the MEP as an adhesin. The positively charged polymer could prevent association of the MEP with the cell surface by binding to the anionic polysaccharide. Poly-L-aspartate may have interfered with the activity of another important component of the binding, such as divalent cations.

The results of the sugar inhibition studies suggest that GlcNAc, Gal, and *N*-acetylneuraminic acid residues may be involved in the interaction of MEP with the cell surface, although the site of action of the inhibitor could not be determined. Vishwanath and Ramphal reported that *N*-acetylneuraminic acid and GlcNAc inhibited adherence of mucoid and nonmucoid strains of *P. aeruginosa* to human tracheobronchial mucin (15). Inhibition by GlcNAc and Gal is consistent with the recent observation that oligosaccharides with a terminal Galβ1-4GlcNAc sequence inhibit the binding of *P. aeruginosa* to mucin (R. Ramphal, N. Houdret, G. Lamblin, G. Strecker, and P. Roussel, Proc. 9th Int. Symp. Glycoconj., abst. no. G131). Thus these sugars may be interfering with attachment of the organisms to the mucous blanket. Recent studies of the adherence of strains of *P. aeruginosa* to glycolipids show that most strains adhere to sialic acid containing glycolipids (N. R. Baker et al., manuscript in preparation). *N*-Acetylneuraminic acid residues on glycolipids or glycoproteins appear to be important for adherence of these organisms.

Since the adhesin is a polysaccharide, there is a possible need for a lectin or divalent cations to link the bacteria and host polysaccharides. A lung lectin capable of binding to MEP has been reported (10). Results of preliminary studies with that lectin have not been conclusive. The two lectins that we tested which enhanced adherence have a specificity for Galβ1 containing di- or trisaccharides. The specificity of PHA is Galβ1-4GlcNAc, which has been proposed as the receptor sequence on mucins for *P. aeruginosa*. *A. hypagea* agglutinin is specific for the terminal Galβ1-3GalNAc sequence, which is the terminal sequence of gangliotetrosylceramide. *Pseudomonas* species bind to this glycolipid, indicating that it may be a receptor on cell surfaces (8). Thus,

PHA may enhance the binding of the bacteria to mucins, whereas *A. hypogea* agglutinin could mediate binding to the cells. How they interact with the bacteria is not clear, but they may associate nonspecifically with the alginate or bind to other polysaccharides on the bacterial cell. The results demonstrate that lectins can mediate the adherence of mucoid strains of *P. aeruginosa* to tracheal cells. The role of lectins in the adherence of mucoid strains of *P. aeruginosa* to the ciliated epithelium needs further study.

The role of divalent cations in the adherence of mucoid strains to the tracheal epithelium is not clear. High concentrations of Ca^{2+} (15 mM) enhanced adherence of mucoid strains seven- to ninefold, but this concentration is higher than reported physiological levels. Mg^{2+} had a less dramatic effect. Since the medium used in the assay contains calcium, we have done the assay in phosphate-buffered saline without calcium and obtained similar results.

We have identified a clear selective advantage of the mucoid organisms which may explain, at least partially, the prevalence of these organisms in CF patients. Attachment of the mucoid variants to cilia may be an important early step in colonization of the lower respiratory tract and in persistence in the lungs of patients with impaired mucociliary clearance. Lectins of host or bacterial origin and divalent cations may be involved in the interaction of MEP with the cell surface. Studies which will define the mechanisms of attachment more clearly are in progress. The MEP also is important in protecting the organisms from phagocytosis (13) and the action of antibiotics (5), thus allowing the organisms to persist for long periods. A more complete understanding of the interaction of the organisms with the tissues they colonize may lead to improved methods of prevention and elimination of the infections.

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