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Pseudomonas aeruginosa Adhesins for Tracheobronchial Mucin

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Mucins of the tracheobronchial tree are preferential sites for adherence and colonization by *Pseudomonas aeruginosa*. They possess specific receptors for this organism that have amino sugars as their principal constituents. Since mucins probably reflect the receptors on the cellular surfaces, we hypothesized that the bacterial adhesins previously shown to mediate the binding of *P. aeruginosa* to cells would also mediate bacterial binding to mucins. We therefore tested the roles of the exopolysaccharide from mucoid strains of *P. aeruginosa* and pili from nonmucoid strains to see whether they are indeed the adhesins for mucins. Using a microtiter plate assay of adherence to mucins, we demonstrated that the mucoid exopolysaccharide bound to mucins and enhanced the adherence of mucoid strains to this substance. Antibodies raised against the exopolysaccharide from a single mucoid strain inhibited the adherence of all mucoid strains tested. Purified pili from nonmucoid strains inhibited the binding of nonmucoid strains but not of mucoid strains. Inhibition of adherence by antibody to pili was quite specific, antibody being able to inhibit only the binding of the homologous nonmucoid strain. These data support our previous observations with tracheal cells, confirming the similarity of the adhesins for respiratory tract cells and the mucins which cover them.

Tracheobronchial mucins and tracheal cells possess receptors for *Pseudomonas aeruginosa* which have been partially characterized (11). For these tracheal cells, the bacterial adhesins that bind to the receptors have been elucidated and appear to be pili for nonmucoid strains (8) and the mucoid exopolysaccharide (MEP) for mucoid strains (5). An important question is whether the same would hold for mucins since mucins often reflect the structure of the cell surface glycoconjugates (12) and thus could serve as a barrier to adherence to tracheal cells.

Using a microtiter plate assay which measures the binding of *P. aeruginosa* to immobilized human tracheobronchial mucin (HTBM), we gathered data which parallel the observations made with acid-injured tracheal cells, i.e., that pili and MEP are respectively responsible for the binding of nonmucoid and mucoid strains of *P. aeruginosa* to mucins.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* 2192, M35, 1, 283, OK, 1244, R1, and T_2A have been described in earlier publications (4–8). The bacteria were grown up overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), washed, and then suspended in phosphate-buffered saline and adjusted by optical density measurements to the approximate inoculum. The exact inoculum was determined by dilution and plating on MacConkey agar (BBL). Between 10^6 and 10^8 CFU/ml were used in the experiments.

MEP, pili, and antisera. MEP was prepared from mucoid strain 2192 as previously described (4). Radiolabeled MEP was prepared by growing mucoid strain 2192 on Trypticase soy agar (BBL) plates containing 20 μCi of ^{14}C-labeled sodium acetate. The labeled MEP was purified by the procedure previously described (4). The purified MEP was dissolved in deionized distilled water before use. Antiserum against the MEP was prepared in rabbits as previously described (4) and was used at a final dilution of 1/200. Incubation of the mucoid organisms with this dilution of the antiserum did not produce macroscopic agglutination or a reduction in the inoculum count which would have indicated microscopic agglutination. Pili from strains 1244 and T_2A and antisera against these pili were the same as those used in a prior study (8). The pili were originally prepared by Charles Brinton, Univeristy of Pittsburgh, Pittsburgh, Pa. The antiserum which was prepared by vaccinating rabbits with purified pilus protein was the same as that used previously (8). This antiserum, used at a final dilution of 1/200, also did not produce macroscopic or microscopic agglutination of the bacteria.

Bacterial adherence assay. The bacterial adherence assay has been described previously (10). In brief, HTBM purified from tracheal aspirates (10) was used to coat the wells of a 96-well microtiter plate by overnight incubation at 37°C. Excess mucin was removed by washing the wells in a microtiter plate washer with phosphate-buffered saline. Bacterial suspensions (100 μl) were then added to the wells and incubated for 30 min at 37°C. Excess nonadherent bacteria were then washed away with the microtiter plate washer. Adherent bacteria were desorbed with a 0.5% Triton X-100 solution and quantitated by diluting and plating a sample of the Triton X-100 solution on MacConkey agar plates. A set of uncoated wells served as controls for background binding on each experiment. Only experiments with little or no background binding were considered valid. This basic method was modified depending on the question being asked.

Effect of purified MEP on adherence of *P. aeruginosa*. In the initial experiments, increasing concentrations of MEP were added to the mucin-coated wells and allowed to incubate at 37°C for 30 min. Excess MEP was then gently washed away, and the bacteria were added to the wells. In addition to measuring the effect of increasing concentrations of MEP on the adherence of the mucoid strain 2192 and nonmucoid revertant strain nM2192, we examined the same effect using

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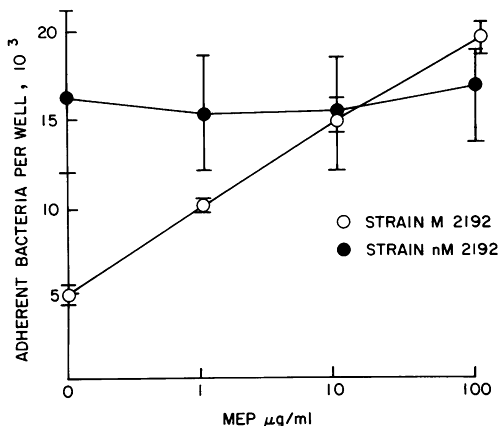

FIG. 1. Effect of increasing concentrations of purified MEP on the adherence of mucoid strain 2192 and nonmucoid strain nM2192 to wells coated with mucin. Inocula were 4×10^6 and 8×10^6 CFU/ml, respectively.

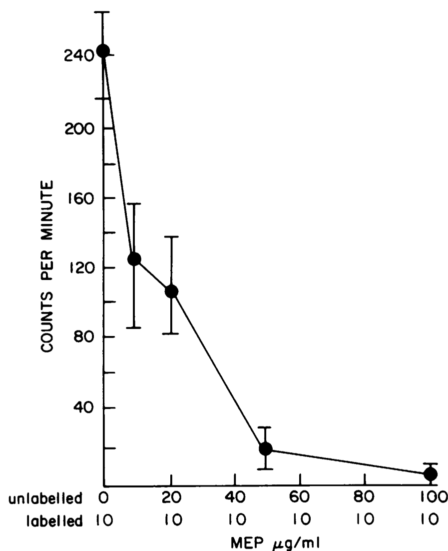

FIG. 2. Competitive binding of a mixture of ^{14}C-labeled and unlabeled MEP to mucin-coated wells.

five more mucoid strains at a fixed concentration of MEP. All the experiments were done three to five times. Unavoidably, the inoculum used from strain to strain differed, despite the use of optical density measurements. Uncoated wells incubated with the highest concentration of MEP (100 µg/ml) were used as controls. These controls showed negligible binding of bacteria, the same as uncoated wells without MEP.

Direct binding of MEP to mucin-coated wells. Radiolabeled purified MEP was added to mucin-coated and uncoated wells to test whether the MEP binds to mucin. A 100-µl sample of the MEP solution containing 3,700 cpm was added to the wells and incubated for 30 min at 37°C. Unbound MEP was then washed away by using a semi-automatic multiple sample precipitator to wash the wells. The wells were then treated with 1% Triton X-100 for 1 h to remove the bound labeled MEP and washed five times with fresh Triton X-100, and the washes were collected and counted in a scintillation counter. Inhibition of the binding of radiolabeled MEP was done by adding increasing quantities of unlabeled MEP to a fixed amount of labeled MEP in a total volume of 100 µl in mucin-coated wells. The wells were then treated as described above.

Effect of anti-MEP antibody on adherence. Rabbit antibody against MEP from mucoid strain 2192 was tested for its ability to inhibit adherence of several mucoid strains of *P. aeruginosa* by incubating the different bacterial strains with the antiserum for 60 min at 37°C. Excess antibody was removed by pelleting the bacteria and washing them gently in phosphate-buffered saline. Preimmune serum from the same immunized rabbit was used to treat the control bacteria. The serum-treated bacteria were then tested in the basic adherence assay.

TABLE 1. Effect of MEP on adherence of mucoid strains of *P. aeruginosa* to HTBM

Strain[a]	No. of adherent bacteria/well (10^2, mean ± SD)		% Increase	*P* value[b]
	No MEP	10 µg of MEP/ml		
M35	34.2 ± 5.7	81.0 ± 40.7	136	<0.01
258	204 ± 89.2	480 ± 52.9	135	<0.001
1	47.5 ± 6.4	80.7 ± 2.7	70	<0.001
283	557 ± 26.5	827 ± 90.9	60	<0.001
OK	264 ± 63.5	560 ± 55.5	112	<0.001

[a] Inocula varied from strain to strain (10^6 to 10^8 CFU/ml).
[b] *P* valuedetermined by Student's *t* test for all experiments.

Effect of pili and antipilus antibody on adherence. Increasing concentrations of pilus protein in 100-µl volumes were added to mucin-coated wells and incubated for 60 min at 37°C. The wells were then washed to remove unbound pilus protein. The bacterial strains were added to the wells, and the rest of the assay was done as described above. After it was ascertained that preincubation of the mucin-coated wells with pili inhibited the adherence of homologous strains, we tested the effects of the pili on the binding of heterologous nonmucoid and mucoid strains, using a fixed concentration of pilus protein. Antipilus antibody against strain 1244 pili at a dilution of 1/200 was tested against nonmucoid and mucoid strains as described above for antiserum to MEP.

RESULTS

Effect of MEP on adherence. The addition of increasing amounts of MEP to the mucin-coated wells followed by incubation with the bacterial strains resulted in increasing adherence of mucoid strain 2192 to the mucin (Fig. 1). The enhancement occurred over a wide range of MEP concentrations. There was no effect on the adherence of nonmucoid revertant strain nM2192 which was derived from the mucoid strain by in vitro passage. The phenomenon of enhancement was also observed with the five other mucoid strains tested (Table 1), using a concentration of 10 µg of MEP per ml. This general phenomenon paralleled our observations with tracheal cells (5).

Interaction of MEP with mucin. The addition of ^{14}C-labeled MEP to mucin-coated and uncoated wells resulted in the binding of the labeled MEP to both types of wells, but the amount bound to mucin-coated wells was almost twice that which bound to the uncoated plastic (252 ± 30 cpm versus 139 ± 22 cpm, $P < 0.01$ by Student's *t* test). The specificity of the binding of MEP to mucin was further investigated by adding an excess of unlabeled MEP to compete with ^{14}C-

labeled MEP. Increasing concentrations of unlabeled MEP mixed with a fixed amount of labeled MEP resulted in almost no binding of labeled MEP when a 10:1 ratio was used (Fig. 2).

Inhibition of adherence by antibody to MEP. Antibody against the exopolysaccharide from mucoid strain 2192 inhibited the adherence of the six mucoid strains tested but not the two nonmucoid strains (Table 2). Inhibition was greatest with homologous mucoid strain 2192 and mucoid strain 1, against which the antibody had its highest titer (4). In our previous study (with tracheal cells) the binding of strain 258 was not inhibited by this antiserum. However, these antibodies do react with strain 258 in other assays (4), albeit to a lesser degree, indicating that our current assay system allowed detection of this immune activity.

Effect of pili and antipilus antibody on adherence. The pretreatment of mucin-coated wells with two different pilus proteins resulted in a dose-dependent inhibition of adherence of both homologous strains (Fig. 3). When one of these pili (strain 1244) was used in inhibition studies with other heterologous *Pseudomonas* strains, it was inhibitory toward nonmucoid strains but not against the three mucoid strains tested (Table 3). Antipilus antibody was very specific in its action, since antibody against pili from strain 1244 inhibited the binding of strain 1244 alone and not the other nonmucoid piliated strains tested (Table 4).

DISCUSSION

P. aeruginosa adheres to tracheal cells by means of pili if the strains are nonmucoid (8) or by MEP if the strains are mucoid (5). Adherence to buccal cells may also be mediated by these same adhesins (3, 13). We tested whether pili and MEP would mediate bacterial binding to HTBM. The data presented in this manuscript do indeed support this contention and define the specificity of the binding by different strains. As before (5), we found that the MEP does not inhibit the binding of mucoid strains when incubated with HTBM before addition of the bacteria, but instead enhances the binding. We have not been able to provide experimental evidence to describe the mechanisms, but offer the explanation that MEP bound to mucins forms a bridge between mucins and the bacteria. It appears that most if not all mucoid strains bind to HTBM by an antigenically related

TABLE 2. Effect of anti-MEP antibodies on adherence of mucoid and nonmucoid strains of *P. aeruginosa* to HTBM

Strain[a]	No. of adherent bacteria/well (10^2, mean ± SD) Preimmune serum	Immune serum[b]	*P* value
Mucoid			
2192	91.7 ± 46.8	9.8 ± 2.5	<0.001
1	292 ± 68.2	24.8 ± 5.3	<0.001
M35	106 ± 15.2	67.0 ± 27.5	<0.001
258	31.5 ± 5.2	17.3 ± 1.7	<0.001
283	200 ± 16.5	128 ± 13.7	<0.001
OK	67.2 ± 0.7	23.0 ± 3.2	<0.001
Nonmucoid			
1244	79.3 ± 6.8	92.8 ± 16.4	NS[c]
nM2192[d]	32.7 ± 4.2	35.7 ± 9.3	NS

[a] Inocula varied from strain to strain (10^6 to 10^8 CFU/ml).
[b] Antibody prepared against strain 2192 MEP.
[c] NS, Not significant.
[d] nM2192 is a nonmucoid revertant of mucoid strain 2192.

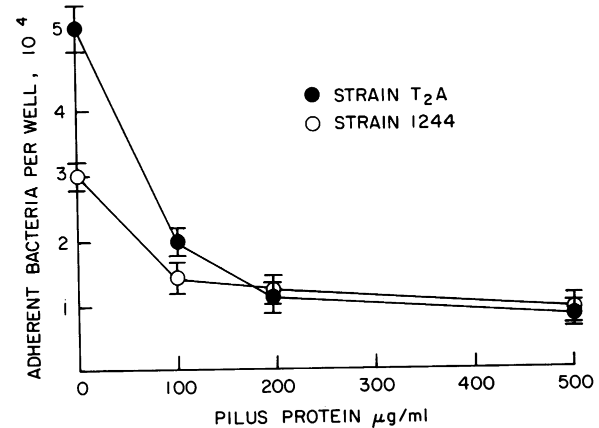

FIG. 3. Effect of purified pilus protein on adherence of homologous piliated nonmucoid strains of *P. aeruginosa*. Inocula: T_2A, 1.7 × 10^7 CFU/ml; 1244, 1.4 × 10^7 CFU/ml.

MEP, whereas nonmucoid strains bind by antigenically dissimilar pili. In addition, there may be subtle differences in the receptors for these two adhesins since the binding of the pilus adhesin to mucin did not inhibit the binding of mucoid strains which adhere via the MEP adhesin. Nor did the presence of MEP affect the binding of nonmucoid strain nM2192 (Fig. 1). This is unexplainable at this time, in light of the fact that we have previously demonstrated that the receptors for the two types of strains both contain *N*-acetylneuraminic acid and *N*-acetylglucosamine (11). We expect that this paradoxical finding will be explained with further studies on isolated receptor oligosaccharides or by a better understanding of the physicochemical steps involved in the adherence of *P. aeruginosa*.

It is now clear that serious consideration must be given to mucins as the important site in colonization of the respiratory tract in cystic fibrosis since these substances cover the surfaces of respiratory tract cells and contain receptors for the adhesins of *P. aeruginosa*. We are unaware of any studies that demonstrate the in vivo binding site of *P. aeruginosa* in the living cystic fibrosis patient. There is one study which demonstrates binding to an alveolar cell in a postmortem specimen (2), but the relevance of these data is no more certain than the binding of *P. aeruginosa* to injured

TABLE 3. Effect of pili on adherence of nonmucoid and mucoid strains of *P. aeruginosa* to HTBM

Strain[a]	No. of adherent bacteria/well (10^2, mean ± SD) Control	Pilus treated[b]	*P* value
Nonmucoid			
T_2A	104.0 ± 5.8	42.0 ± 9.2	<0.001
R1	357.0 ± 9.6	203.0 ± 31.0	<0.001
nM2192[c]	28.0 ± 3.1	13.8 ± 4.5	<0.001
Mucoid			
2192	50.0 ± 19.0	59.0 ± 13.0	NS[d]
258	213.0 ± 5.8	211.0 ± 4.9	NS
M35	28.3 ± 5.1	27.3 ± 8.6	NS

[a] Inocula varied from strain to strain (10^6 to 10^8 CFU/ml).
[b] Wells exposed to 250 µg of pilus protein per ml from strain 1244.
[c] Piliated nonmucoid revertant from mucoid strain 2192.
[d] NS, Not significant.

TABLE 4. Effect of antipilus antibody on adherence of nonmucoid strains of *P. aeruginosa* to HTBM

Strain[a]	No. of adherent bacteria/well (10^2, mean ± SD)		*P* value
	Control	Antibody treated	
1244	126.8 ± 7.3	56.5 ± 6.2	<0.001
R1	88.8 ± 4.3	88.2 ± 5.3	NS[b]
nM2192[c]	92.5 ± 3.6	92.0 ± 5.0	NS
T_2A	86.0 ± 5.2	84.3 ± 3.3	NS

[a] Inocula varied from strain to strain (10^7 to 10^8 CFU/ml).
[b] NS, Not significant.
[c] Piliated nonmucoid revertant from mucoid strain 2192.

cells (6). Thus, until the questions relating to the actual in vivo binding sites are answered, we believe that mucins of the tracheobronchial tree are strong candidates as the primary site of colonization in this disease process. The demonstration of bacterial adhesin specificity for mucins supports our contention. However, in other diseases such as nosocomial pneumonias due to *P. aeruginosa*, the cell injury produced by endotracheal intubation (9), acid aspiration (6), or cytotoxic chemotherapy may very well predispose to transient colonization of cells and hence acute infection. In the cystic fibrosis respiratory tract, however, chronic colonization is seen, and this process may be best explained by adherence to mucins mediated by specific receptor-adhesin mechanisms.

The study of adhesins carries with it the implication that interfering with the initial adherence process could result in prevention of the disease. In the situation of *P. aeruginosa* and the respiratory tract, a vaccine designed to elicit antibodies that prevent adherence would seem desirable. Antibody against MEP would suffice since antibody against one strain is antiadhesive for all other strains. However, nonmucoid strains have been reported to be the initial colonizers in cystic fibrosis and are also found in the later stages of colonization, and thus the problem may not be solved by this approach. Interruption of the initial colonization process may require antibodies directed at the pili which are involved in the adherence of these early colonizing nonmucoid strains. These antibodies are less attractive than antibodies specific to MEP, since the pili show antigenic heterogeneity, whereas the MEP appears to show antigenic homogeneity in regards to inhibition of binding by antibodies. Notwithstanding problems of antigenicity, there are questions about whether antibodies within the respiratory tract are functional. In addition to defects in their opsonizing ability in cystic fibrosis (1), we have recently uncovered defects in their ability to prevent adherence when compared with serum antibody (R. Ramphal, C. Guay, J. Saunders, and G. B. Pier, Clin. Res. 34:530, 1986). One should, however, make the distinction that these defects in antibody function occur after chronic colonization has begun. It is possible that preexisting antibody induced by immunization would be able to prevent adherence and hence colonization. Thus, the ultimate importance of adherence and the role for antibodies which interrupt adherence must be studied in a vaccine situation, in which an appropriate vaccine is tested for the ability to prevent the chronic colonization of cystic fibrosis patients by *P. aeruginosa*.

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