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Evidence for Mucins and Sialic Acid as Receptors for *Pseudomonas aeruginosa* in the Lower Respiratory Tract

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The nature of the receptors for mucoid and nonmucoid *Pseudomonas aeruginosa* was investigated by using adherence to injured tracheal epithelium as a model. Bovine submaxillary mucin and crude rat tracheal mucin inhibited the adherence of both types of *P. aeruginosa*. Among the sugars present in these mucins only *N*-acetylneuraminic acid inhibited adherence. Inhibition of adherence probably involved the binding of *N*-acetylneuraminic acid to the bacterial cells and not to the tracheal cells. The mucoid strain appeared to be much more sensitive to inhibition by *N*-acetylneuraminic acid. Periodate oxidation and cholera filtrate also reduced the adherence of both strains, but *Clostridium perfringens* neuraminidase treatment did not alter adherence. A nonmucoid isogenic mutant of an unstable mucoid strain was also inhibited by *N*-acetylneuraminic acid. These data suggest that the receptor for *P. aeruginosa* is a sialic acid moiety on cell surfaces or in mucins.

Respiratory tract infection by *Pseudomonas aeruginosa* is a major problem in hospitalized patients and in persons with cystic fibrosis. In both situations adherence to respiratory tissue is likely to play a critical role in the bacteria-host interaction.

Some progress has been made in understanding mechanisms involved in the adherence of *P. aeruginosa* to host tissues, but the picture is far from clear. At the present time several models to study adherence of this organism have been described (8, 13, 15), but most of the work on the mechanisms involved in adherence has utilized buccal cells. Woods et al. (24), using human buccal cells, have shown that pili mediate the adherence of rough *P. aeruginosa* strains. These investigators have also reported some elegant studies showing that adherence of this organism to buccal cells is correlated with the loss of fibronectin from cell surfaces (23). They did not identify the nature of the receptor on the buccal cell, but they ruled out sialic acid as a possible receptor (23). One paradoxical finding was that the mucoid strains which are responsible for the state of chronic colonization in cystic fibrosis adhered poorly to buccal cells (22), in spite of the fact that mucoid strains possess pili. Thus, there are gaps in our knowledge of the receptor for nonmucoid *P. aeruginosa*, and our knowledge of both adhesin and receptor for mucoid *P. aeruginosa* is incomplete.

In the accompanying paper we describe the use of the acid-injured mouse trachea as a model to study the adherence of both mucoid and nonmucoid strains of *P. aeruginosa* (15). In this model both mucoid and nonmucoid *P. aeruginosa* organisms adhered to injured cells, whereas *Escherichia coli* and *Klebsiella pneumoniae* did not. In addition, we observed that mucoid strains of *P. aeruginosa* appeared to adhere to mucin strands in uninjured tracheas. The present study has utilized these observations and this model to elucidate possible receptors for mucoid and nonmucoid strains of this organism. We found that mucins and sialic acid inhibited the adherence of mucoid and nonmucoid strains of *P. aeruginosa* to injured tracheal cells and thus may serve as receptors for this organism in the lower respiratory tract.

MATERIALS AND METHODS

Bacteria. Three strains of *P. aeruginosa* were used: a nonmucoid strain isolated from a sputum culture of a hospitalized patient and two mucoid strains isolated from cystic fibrosis patients. The nonmucoid strain and one of the mucoid strains (a stable mucoid strain provided by Lee Boyd, University of Texas at San Antonio) were used in all the experiments, except the one which dealt with the adherence of an isogenic nonmucoid variant of the unstable mucoid strain. The unstable mucoid strain was obtained from the Clinical Laboratories of Shands Teaching Hospital, Gainesville. All strains had been isolated at least 6 months before use. They were kept on McConkey agar plates at room temperature and subcultured monthly. Bacteria were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C under

static growth conditions. After 18 to 24 h of incubation, the organisms were centrifuged at 10,000 × g for 15 min, washed with phosphate-buffered saline (PBS), pH 7.2, and suspended as per the experimental protocol. Inocula were quantitated by optical density measurement and confirmed by viable counts.

Tracheal organ culture preparation. Tracheal organ cultures were prepared from 6- to 8-week-old CD-1 mice as described in the accompanying paper (14). In brief, the tracheas were injured by exposing the luminal surface to 0.1 N hydrochloric acid (pH 1.48) for 15 min and sectioning the tracheas into pieces consisting of two to four rings. Control mice were treated with PBS instead of acid.

Adherence testing. Three or four pieces of tracheal tissue were placed in a small plastic petri dish, to which 1 ml of the test bacterial suspension had been added. The liquid always covered the tissues. The petri dish was incubated at 37°C for 1 h. After the incubation, each piece of tissue was rinsed gently in three changes of PBS and then put in 3% glutaraldehyde for fixation. After a minimum of 24 h of fixation, the tracheal samples were mounted, critical point dried, and coated with gold palladium for scanning electron microscopic examination as previously described (15).

Quantitation of adherence. Adherence was quantitated by means of direct count using the scanning electron microscope as described in the accompanying paper (14). Briefly, we counted the number of bacteria in a microscopic field of a fixed size. The size of the field was fixed by setting the working distance from the sample to the bottom pole piece of the objective lens (10 mm), fixing the angle from which the sample was viewed (30°), and using the same magnification (ca. 3,6000×) each time. The number of bacteria within a field was counted, and the field was randomly changed. Six randomly selected fields were counted on each of three pieces of tracheal tissue, and seven fields were counted on the fourth piece, for a total of 25 fields. The data are reported as the mean number of bacteria ± 1 standard deviation adherent to 1,000 μm^2 of the tracheal surface.

Inhibition by mucin. Crude tracheal mucin was obtained from Sprague-Dawley rats. The rats were anesthetized with sodium pentobarbital, and the chest cavity was opened to expose the lungs and heart. The animals were exsanguinated by cardiac puncture, and the tracheas were excised from the larynx to below the tracheal bifurcation. Two milliliters of PBS was repeatedly passed through the tracheal lumen to wash out tracheal mucins. The slightly turbid mucin suspensions from five rats were collected, pooled, and centrifuged at 300 × g for 5 min to remove cells. The presence of mucins in each sample was ascertained by observing the characteristic ferning appearance of mucins when placed on a microscopic slide. This crude mucin preparation was quantitated by ascertaining the protein concentration by the Bradford method (3) and was adjusted to a concentration of 100 μg of protein per ml. In some experiments, bovine submaxillary mucin was obtained from Sigma Chemical Co. (St. Louis, Mo.) and used at a concentration of 200 μg of protein per ml.

To test for inhibition of adherence, 1 ml of the mucin preparation was incubated with 1 ml of the bacterial suspension for 30 min at 37°C. The mixture was centrifuged at 10,000 × g for 15 min, and the bacterial pellet was washed three times in PBS and then suspended in 1 ml of PBS. A bacterial suspension to be used as control was treated in the same way, but PBS was added to the bacteria instead of mucin. The bacteria thus prepared were incubated with the tracheal pieces as described above. In another set of experiments the mucins were added to petri dishes containing the bacteria and tracheal pieces, instead of being preincubated with the bacteria.

Effect of sugars and sialic acid on adherence. D-(+)galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, L-(−)fucose, and *N*-acetylneuraminic acid (NANA)—the five sugars present in respiratory mucin (7)—were used in inhibition experiments. D-(+)mannose, a sugar generally not present in respiratory mucins (7) but known to serve as a receptor for some *E. coli* strains (12), was also tested. These reagents were purchased from Sigma Chemical Co., St. Louis, Mo., and were used at a concentration of 5 mg/ml, except for NANA, which was used at 0.01 to 1 mg/ml. Bacterial strains were suspended in the sugars and incubated at 37°C with the tracheal pieces. The control bacteria were incubated with PBS instead of a sugar moiety. In one set of experiments the tracheal rings were preincubated with NANA and then washed before use in adherence studies. In the same experiment a bacterial pellet was suspended in NANA, incubated for 0.5 h at 37°C, washed three times, and suspended in PBS before use in adherence studies.

Periodate treatment. Acid-injured tracheal rings were incubated with 10 mM sodium metaperiodate (Sigma) in PBS for 20 min at 37°C. After treatment the

TABLE 1. Effect of rat tracheal mucin on adherence

P. aeruginosa[a]	No. of bacteria per 1,000 μm^2, mean ± SD			% Inhibition	*P* value[b]
	Control	Mucin-treated bacteria	Mucin in plate		
Mucoid	14.9 ± 8.0	1.9 ± 1.4		87	<0.001
Nonmucoid	14.9 ± 9.6	2.7 ± 2.7		75	<0.001
Mucoid	13.0 ± 5.9		2.4 ± 1.6	82	<0.001
Nonmucoid	16.5 ± 5.6		4.2 ± 2.6	75	<0.001

[a] Inoculum: 2×10^7 CFU/ml.
[b] Student's *t* test.

TABLE 2. Inhibition of adherence of *P. aeruginosa* by sugars

Prepn[a]	No. of bacteria per 1,000 μm^2 mean ± SD (% of control)	
	Mucoid[b]	Nonmucoid[c]
Control	26.6 ± 5.0	10.0 ± 3.4
NANA	0.4 ± 0.6 (1.9)	0.8 ± 0.7 (8)
D-(+)Galactose	25.6 ± 6.8 (124)	10.6 ± 2.2 (106)
L-Fucose	27.6 ± 6.0 (134)	10.0 ± 3.8 (100)
N-Acetyl-glucosamine	40.1 ± 9.1 (195)	10.7 ± 3.2 (107)
N-Acetyl-galactosamine	24.5 ± 4.4 (119)	9.4 ± 3.6 (94)
D-(+)Mannose	37.2 ± 10.9 (180)	11.8 ± 3.3 (118)

[a] NANA was used at 1 mg/ml; other sugars were used at 5 mg/ml.
[b] Inoculum: 10^8 CFU/ml.
[c] Inoculum: 10^7 CFU/ml.

rings were rinsed with PBS and used in adherence studies. Untreated, acid-injured rings were used as controls.

Neuraminidase treatment. *Clostridium perfringens* neuraminidase (Sigma types V and VI) was dissolved in 0.05 M acetate buffer (pH 5.5). Cholera filtrate was dissovled in sterile distilled water as recommended by Sigma. Both neuraminidases were used at concentrations of 1 U/ml, but the cholera filtrate contained less than 0.1 U of neuraminidase activity in 5 ml. One milliliter of each solution was used to treat the injured tracheas for various periods of time at 37°C. After treatment, tracheas were rinsed with PBS before adherence studies. Acid-injured but untreated tracheas served as controls. Before use, the activity of each neuraminidase and the cholera filtrate was verified by measuring the release of sialic acid from bovine submaxillary mucin (20). Each preparation was active.

Measurement of sialic acid release from tracheas. The ability of the type VI neuraminidase to release sialic acid from the normal and injured mouse trachea was measured. Normal and acid-injured tracheas were rinsed with PBS to remove mucins. All tracheas were trimmed to the same length (ca. 6 mm) then cut into pieces of two to four rings. Each set of tracheal rings was treated separately with neuraminidase to release sialic acid. Sialic acid was also released from another set of normal and acid-injured tracheas by heating at 80°C for 1 h in 0.5 ml of 0.05 N H_2SO_4. The amount of sialic acid released by both methods was measured by the thiobarbituric acid assay (20).

RESULTS

Effects of mucins on adherence. Both rat tracheal mucin and bovine submaxillary mucin inhibited the adherence of the mucoid and nonmucoid strains *P. aeruginosa*. Inhibition occurred regardless of whether the bacteria were pretreated with the mucins, washed, and suspended in PBS or whether the mucins were added to the dishes containing the bacteria and tracheas. Inhibition was generally 75% or more for each type of mucin and for each strain. However the degree of inhibition appeared to be greater with the bovine submaxillary mucin, which was used at a higher concentration. The data for inhibition by rat tracheal mucin are shown in Table 1.

Effect of sugars and sialic acid on adherence. Since both of these mucins inhibited adherence of the two types of *P. aeruginosa*, it was possible that one of the sugars in these mucins was occupying the attachment site(s) on the bacteria. Both respiratory tract mucin and bovine submaxillary mucin contain at least five sugars, but not mannose (7, 19). The five sugars and mannose were therefore tested for their ability to inhibit adherence of *P. aeruginosa* (Table 2). Only NANA inhibited the adherence of these strains. The use of much lower concentrations of NANA also inhibited adherence (Table 3). The mucoid strain demonstrated exquisite sensitivity to as little as 10 μg of NANA per ml, and the nonmucoid strain demonstrated a clear dose response. There were two main possibilities to explain inhibition of adherence by NANA: (i) that NANA was adhering to the bacterial adhesin(s) and blocking the attachment site on the adhesin, or (ii) that NANA was preventing adherence by occupying sites on the trachea. To test these two possibilities, bacteria were treated with 1 mg of NANA per ml at 37°C for 30 min and then washed before use with untreated tracheas, and a set of tracheas were treated with NANA in the same way for use with untreated bacteria. Adherence of NANA-treated bacteria to untreated tracheas and adherence of untreated bacteria to NANA-treated tracheas were then compared with controls. The controls consisted of untreated bacteria and tracheas with and without NANA added to the petri dish. The NANA-treated tracheas behaved like the untreated control (i.e., there was no difference in adherence), whereas the NANA-treated bacteria were inhibited and were similar to NANA added to the organ cultures (Table 4). Clearly the ability of NANA to inhibit adherence was mediated by binding of NANA to the bacteria and not by binding to the trachea.

TABLE 3. Dose response of inhibition of adherence by NANA

NANA concn (mg/ml)	No. of bacteria per 1,000 μm^2, mean ± SD (% inhibition)	
	Mucoid[a]	Nonmucoid[b]
0	54.1 ± 11.0	44.5 ± 5.6
1	1.7 ± 1.0 (96)	5.4 ± 4.3 (88)
0.1	1.5 ± 0.9 (97)	9.3 ± 4.9 (80)
0.01	1.7 ± 1.0 (96)	22.8 ± 10.6 (49)

[a] Inoculum: 10^8 CFU/ml.
[b] Inoculum: 10^8 CFU/ml.

TABLE 4. Inhibition by binding of NANA to bacteria[a]

P. aeruginosa	No. of bacteria per 1,000 μm², mean ± SD			
	Control (no NANA)	NANA-treated treachea	NANA-treated bacteria	NANA in organ culture
Nonmucoid	15.3 ± 8.1	14.0 ± 8.0	0.2 ± 0.4	0.1 ± 0.3
Mucoid	10.3 ± 5.5	7.7 ± 3.7	0.1 ± 0.3	0.1 ± 0.2

[a] Inoculum: 10^7 CFU/ml.

Effect of periodate oxidation and neuraminidase. To obtain further evidence for the role of NANA on the tracheal surface as the receptor residue for *P. aeruginosa*, the tracheal pieces were treated with 10 mM periodate for 20 min and with the neuraminidases from *C. perfringens* (Sigma types V and Vi) and *Vibrio cholerae* (cholera filtrate). Sodium metaperiodate reduced adherence significantly, as did the cholera filtrate (Table 5), but we were unable to demonstrate any reduction in adherence by treating with *C. perfringens* neuraminidase. The concentration used was as high as 1 U of the type VI enzyme for 15 to 20 min of treatment. Both the cholera filtrate and *C. perfringens* neuraminidase cleaved sialic acid from bovine submaxillary mucin in preliminary testing; hence the lack of an effect with *C. perfringens* neuraminidase was puzzling. We therefore tested the ability of the *C. perfringens* type VI enzyme to release sialic acid from tracheal tissue and compared the amount released to that removed by 0.05 N H_2SO_4. Acid treatment released roughly the same amount of sialic acid from normal tracheas as from acid-injured tracheas, whereas neuraminidase treatment (0.2 U for 15 min at 37°C) did not release detectable amounts of sialic acid. These results also indicate that the original acid injury did not release significant amounts of sialic acid, if any.

Effect of NANA on a nonmucoid variant. During in vitro passage and culture of mucoid *P. aeruginosa*, it is common for isogenic nonmucoid variants to arise spontaneously. We tested the ability of such a variant from the unstable mucoid strain to adhere to the injured trachea and the ability of NANA (1 mg/ml) to inhibit this variant. The variant adhered to the tracheal surface and was also inhibited by NANA.

DISCUSSION

It is thought that an understanding of the process by which bacteria adhere to tissues holds promise for the prevention or modification of infectious diseases. This may be possible with the use of vaccines against bacterial adhesins or the use of receptor analogs to inhibit bacterial adhesion. The former principle has been successfully applied in veterinary medicine (16), but to our knowledge no studies successfully applying this principle to human bacterial infections have been published. With regard to the use of receptor analogs, work is also in its early stages. However, the feasibility of this principle has been demonstrated by Aronson et al. (1) and Svanborg-Edén et al. (18), who showed that methyl alpha-D-mannopyranoside and globotetraose inhibit colonization of the urinary tract by *E. coli*. Numerous studies have identified the receptors for a wide variety of bacteria (2), but the receptor(s) for *P. aeruginosa* has not been identified. The results of this investigation provide several lines of evidence that support our suggestion that sialic acid and mucins may serve as receptors for this organism.

Rat tracheal mucin and bovine submaxillary mucin both inhibited bacterial adherence to injured cells. The fact that inhibition occurred when the mucins were added to the incubation mixture as well as when the bacteria were treated with the mucins and washed supports an interpretation that some component of the mucins was tightly bound to the bacterial adhesin. There is a precedent for mucins serving such a role: Williams and Gibbons (21) demonstrated that salivary glycoproteins inhibited the adherences of *Streptococcus sanguis* to buccal cells. Furthermore, the inhibition of adherence to cells by mucins is consistent with the observation that *P. aeruginosa* adheres to tracheal mucins (14). It should be kept in mind that we used a crude rat tracheal mucin preparation which could have contained other substances. We doubt, however, that it could have contained antibody against our *P. aeruginosa* strains. Further work with individual mucin fractions should yield information about the macromolecule involved.

Of the sugars present in bovine submaxillary and respiratory mucins, only NANA, which is a component of these mucins (7, 19), inhibited the adherence of *P. aeruginosa*. This inhibition was achieved by comparatively low doses of NANA (10 μg/ml or 30 nM); however, it should be noted

TABLE 5. Effect of periodate and cholera filtrate on adherence

P. aeruginosa	No. of bacteria per 1,000 μm², mean ± SD			
	Control	Periodate treatment	Cholera filtrate	% Reduction
Mucoid[a]	11.5 ± 4.7	0.2 ± 0.4		98
	11.4 ± 1.6		0.8 ± 0.9	93
Nonmucoid	42.3 ± 5.5[b]	10.3 ± 3.2		75
	6.0 ± 1.8[c]		0.2 ± 1.6	97

[a] Inoculum: 10^7 CFU/ml.
[b] Inoculum: 10^8 CFU/ml.
[c] Inoculum: 10^7 CFU/ml.

that this represents exposure of 10^8 bacteria to 2 $\times$ 10^{16} molecules of NANA. Since it was possible that NANA was acting as a nonspecific inhibitor of adherence (4) when added to organ culture dishes, treating the bacteria with NANA and then washing them (Table 4) demonstrated that NANA probably inhibited adherence by binding tightly to the bacteria.

Periodate treatment oxidizes α glycols (6). The reduction of adherence after periodate treatment supports the role of NANA as a receptor, although other sugars are also oxidized by this treatment. The reduction in adherence after treatment with cholera filtrate adds more evidence to NANA being the receptor moiety. Of interest, *C. perfringens* neuraminidase did not reduce adherence, but this enzyme also failed to cleave NANA from the mouse trachea. This discrepancy may be explained by the observation that some sialic acid-containing glycoconjugates are resistant to hydrolysis by *C. perfringens* neuraminidase, except under very strict conditions; e.g., G_{M1} and G_{M2} gangliosides require pH 4.0 in the presence of taurocholate at 37°C for 16 h (10).

We also examined the adherence of an isogenic nonmucoid variant of a mucoid strain, and we found that it also was inhibited by NANA. These strains generally appear on in vitro subculture. Their clinical significance is unclear, but it appears that they also adhere to the same receptor.

It should be pointed out that the evidence we present for sialic acid as the receptor for this organism runs contrary to the opinion of Woods et al. (23). However, these authors based their conclusions on the observation that there was less sialic acid recovered from the buccal cells of patients who were prone to *P. aeruginosa* attachment. They did not perform direct competition experiments with sialic acid or degrade sialic acid residues to make their point. It is possible that the discrepancy between their impressions and ours is based on the difference in cell type used (buccal versus tracheal) or species (human versus mouse).

There is precedent that sialic acid may act as a receptor for several organisms. For some time now *Mycoplasma pneumoniae* has been known to bind to a sialic acid-containing glycoconjugate (17); more recently, Lindahl et al. (11) have reported that the colonization factor antigen on enterotoxigenic *E. coli* is a sialic acid-specific lectin. Sialic acid is also part of the receptor for *S. sanguis* on human salivary mucin (9). It is interesting to speculate on the role of adherence to sialic acid and mucins in the pathogenesis of respiratory tract infections. Mucins are present on almost all mucosal surfaces; hence, the true role of adherence to these substances may be defensive, i.e., they may protect the underlying cell surfaces from microbial attachment. It can then be envisaged that if the mucins with adherent bacteria are not cleared, e.g., mucin bound to teeth, or stagnant as in cystic fibrosis, then adherence may lead to bacterial colonization and proliferation on that particular surface. Thus, mucin may serve a pathogenetic role rather than a protective one. The possibility that this is the state of affairs in cystic fibrosis and chronic bronchitis should be entertained.

This study raised some puzzling questions. Sialic acid is normally present on all eucaryotic cell surfaces (5), yet *P. aeruginosa* will not adhere to these surfaces without injury (13–15) or trypsinization (8). In our model we have no explanation, but we speculate that, besides the presence of sialic acid, some other physical or chemical change is required for adherence. The explanation for adherence to the buccal cell is that fibronectin is removed to facilitate adherence (23); we do not know whether this mechanism is operative in the injured trachea. At this time we also do not know the nature of the sialic acid-containing glycoconjugate to which this organism attaches on cell surfaces or the nature of the adhesin which it uses to adhere to this surface.

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