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Role of *Pseudomonas aeruginosa* Mucoid Exopolysaccharide in Adherence to Tracheal Cells

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The mucoid exopolysaccharide of *Pseudomonas aeruginosa* is thought to confer antiphagocytic properties on mucoid strains of *P. aeruginosa*, thus allowing them to persist in the respiratory tract. It has also been speculated that the mucoid exopolysaccharide may be the adhesin for mucoid strains, but proof is lacking. We studied the role of the mucoid exopolysaccharide in adherence of mucoid strains in competitive experiments with purified mucoid exopolysaccharide, by measuring the binding of ^{14}C-labeled mucoid exopolysaccharide to injured tracheas and testing whether an antibody against the major epitope of the mucoid exopolysaccharide inhibits adherence of these organisms. Our data show that the purified mucoid exopolysaccharide increased the adherence of four of the mucoid strains tested (by 50 to 300%; $P < 0.001$) instead of inhibiting adherence. Radiolabeled mucoid exopolysaccharide bound much better to injured tracheal cells than to normal tracheal cells ($P < 0.001$), and antibody against the antigen of strain 2192, the strain from which mucoid exopolysaccharide was prepared, inhibited the adherence of four of five mucoid strains but not the strain lacking this antigen. This antibody also failed to inhibit a nonmucoid revertant from strain 2192, which was previously shown to be inhibited by pili. These data strongly support the thesis that the mucoid exopolysaccharide is the adhesin for mucoid strains of *P. aeruginosa*.

The variety of important roles ascribed to the mucoid exopolysaccharide of *Pseudomonas aeruginosa* provides some basis for understanding why mucoid strains are not effectively cleared by the host immune system in cystic fibrosis. The exopolysaccharide has been reported to be antiphagocytic (12), to inhibit opsonophagocytosis mediated by an antibody against the cell wall lipopolysaccharide (1), and to increase resistance of mucoid strains to coating by antibody (8). In addition to these anti-immune properties, the role of adhesin for mucoid strains has also been ascribed to exopolysaccharide (6). Microscopic evidence provided by Costerton et al. (6) suggests that mucoid strains may adhere by glycocalyxlike material, but these studies do not provide direct experimental proof, and a role for pili cannot be excluded on the basis of these studies.

We investigated the acid-injured murine trachea as a model to study the interaction of mucoid strains with tissues more directly (10). With this model, we were previously able to show that purified pili inhibit the adherence of nonmucoid strains of *P. aeruginosa*, including those derived isogenically from mucoid strains. Pili, however, had no effect on the adherence of mucoid strains (11). With this same model, we now report studies which support a role for the mucoid exopolysaccharide as the adhesin for mucoid strains.

MATERIALS AND METHODS

Bacterial strains. Mucoid strains of *P. aeruginosa* were all isolated from cystic fibrosis patients and stored on MacConkey agar or Desoxycholate-Citrate Agar (BBL Microbiology Systems, Cockeysville, Md.) with 0.054% thiosulfate. These strains have been used in previous studies (9, 10). Clones of mucoid colonies were serially passaged to select fairly stable mucoid isolates. A nonmucoid revertant was selected from strain 2192 in the same manner. For use in the adherence model, strains were grown overnight in Trypticase soy broth (BBL), washed with phosphate-buffered saline (PBS), and adjusted by optical density measurements to the approximate inoculum. The exact inoculum was determined by plating on MacConkey agar. The organisms were generally used at concentrations between 10^7 and 10^8 CFU/ml.

Antigen and antiserum. The mucoid antigen was prepared from strain 2192 as previously described (9). The properties of the mucoid exopolysaccharide of strain 2192 have been previously characterized (9). Radiolabeled exopolysaccharide was prepared by growing mucoid strain 2192 on Trypticase soy agar (BBL) plates containing 20 μCi of ^{14}C-labeled sodium acetate. The exopolysaccharide was then prepared as described previously (9). For use in the adherence experiments, the antigen was dissolved in deionized distilled water. Antiserum against the nonlabeled exopolysaccharide from strain 2192 was raised in rabbits as previously described (9). This antiserum gave a single precipitin line on Ouchterlony immunodiffusion when tested against either crude mucoid exopolysaccharide preparations or purified mucoid exopolysaccharide antigen. The antibody was shown to react with strains 1 and 2192 at high dilution and strain 258 only at low dilution in a hemagglutination assay (9). In the present study, heat-inactivated antiserum was used at a final dilution of 1/100. This dilution did not produce macroscopic or microscopic agglutination of whole cells of any strains.

Adherence assay. Preparation of tracheal organ cultures from mice has been described previously (10). Adherence testing and quantitation were also done as previously described (10). Modifications of these basic procedures are stated under specific experiments. The effect of the mucoid exopolysaccharide on adherence of different mucoid strains of *P. aeruginosa* was tested as follows. In the initial experiments, 1,000 μg of the exopolysaccharide was incubated with the injured tracheal rings for 1 h at 37°C, the rings were

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rinsed, and then the test bacteria were incubated with the rings for 1 h. In other experiments, the exopolysaccharide was mixed with the bacteria and then incubated with the tracheas. The results with both approaches were the same. Controls for these experiments consisted of tracheas exposed to PBS for 1 h and then exposed to the test strains for 1 h at 37°C. After incubation with the bacteria, the tracheas were rinsed three times with PBS and then fixed in 3% glutaraldehyde for quantitation by scanning electron microscopy as previously described (10). Each experiment was done at least three times.

In related experiments with only strain 2192, we tested the effect of different exopolysaccharide concentrations on adherence with a fixed bacterial concentration. We also tested the effect of a fixed concentration of exopolysaccharide against various bacterial inocula. These experiments were done to rule out either a bacterial or a polysaccharide concentration-dependent effect.

Binding of purified exopolysaccharide to tracheal tissue. The ability of the exopolysaccharide to bind directly to injured tracheal tissue was tested with use of the ^{14}C-labeled exopolysaccharide. The polysaccharide had a specific activity of 500 cpm/μg. The experiments were done after first injuring the tracheas as described previously (10), but instead of removing the tracheas from the dead mice, the tracheas were filled with 50 μl of the dissolved exopolysaccharide containing 5,000 cpm (10 μg) of exopolysaccharide. The exopolysaccharide was allowed to remain in contact with the tracheal lumen for 1 h, and then PBS was passed through the trachea to wash out the unbound exopolysaccharide. The tracheas were then removed from the animals, and the tracheal length from the larynx to the tracheal bifurcation was excised. The external tracheal surface was then rinsed with PBS to minimize any external contamination by ^{14}C-labeled exopolysaccharide. The tracheas were cut into smaller pieces and solubilized separately with an NCS tissue solubilizer (Amersham-Searle, Arlington Heights, Ill.) before being counted in a scintillation counter. The controls for this experiment consisted of mice whose tracheas were not acid injured but were exposed to the ^{14}C-labeled exopolysaccharide. Two experiments were performed, each with three animals per group.

Effect of antibody against mucoid exopolysaccharide on adherence of mucoid strains. The antibody prepared in rabbits against mucoid antigen from strain 2192 was tested for its ability to inhibit adherence of different mucoid strains and the nonmucoid revertant of 2192 (NM2192). Before dilution, the antibody had titers of 1:120 against the exopolysaccharide from homologous strain 2192 and heterologous strain 1 and 1:16 against heterologous strain 258 in the passive hemagglutination assay (9). The antibody titers against the polysaccharides from strains M35 and OK are unknown since we did not prepare the exopolysaccharides from these strains. The various bacterial strains were incubated with the antiserum for 1 h, and the mixture was added to the tracheal rings for the basic adherence assay. In initial studies, preimmune rabbit serum was tested against strains 2192 and M35 as a control treatment, but adherence values were no different from those found in bacteria treated with PBS; hence, PBS was used as a control in all subsequent experiments.

RESULTS

Effect of exopolysaccharide on adherence. Exopolysaccharide from strain 2192 was used at a concentration of 1,000 μg/ml in the first series of experiments. The inocula of the strains differed from day to day and from experiment to experiment; hence, it was not possible to compare the effects of the polysaccharide on the different strains. In some cases, low-input inocula were purposely used (e.g., strains M35 and OK) because the number of bacteria in the test part of the assay was too large to allow enumeration by scanning electron microscopy. The overall effect was surprising (Table 1); instead of inhibition of adherence, as is the case when a putative adhesin competes with a bacterium (2), we noted a striking enhancement of adherence with all four mucoid strains. This enhancement proved to be the reason for the need to use lower-input inocula of strains M35 and OK (i.e., a two- to fourfold enhancement of adherence of these strains).

TABLE 1. Enhancing effect of exopolysaccharide on adherence of mucoid *P. aeruginosa* to injured mouse trachea

Strain[a]	No. of bacteria per 1,000 μm² (mean ± SD)		*P* value[b]
	Control	Test	
2191	24.4 ± 4.6	37.5 ± 6.1	<0.01
1	21.4 ± 3.9	37.3 ± 5.5	<0.001
M35	4.5 ± 2.1	17.7 ± 8.6	<0.001
OK	7.8 ± 4.3	17.3 ± 7.5	<0.001

[a] Inocula for each strain: 2192, 9.8×10^7; 1, 7×10^7; M35, 1.2×10^8; OK, 1×10^8 CFU/ml.
[b] One of three separate experiments. *P* value determined by Student's *t* test.

When strain 2192 was used as the adhering organism, we could vary the concentration of the exopolysaccharide over 100-fold without a significant change in adherence (Table 2). The specificity of this enhancement for the injured cell is also evident since there was no significant adherence or enhancement of adherence of strain 2192 to uninjured cells when 1,000 μg of the polysaccharide was used (Table 2). Adherence was also examined with different inocula of strain 2192 and a constant amount of exopolysaccharide. We found that enhanced adherence occurred independently of the inocula of input bacteria used (Table 3).

Binding of ^{14}C-labeled exopolysaccharide to tracheal cells. We examined the direct binding of radiolabeled exopolysaccharide to the injured trachea. There was significantly ($P < 0.001$ by Student's *t* test) more binding of exopolysaccharide to the injured tracheal cells (mean ± standard deviation, 303.4 ± 80.1 cpm bound per trachea) than to the uninjured cells (126.7 ± 38.2 cpm bound per trachea). The uninjured tracheas are fully ciliated, whereas the injured tracheas are devoid of intact cilia (10); hence, the counts found on uninjured cells could represent some trapping of the material. The amount of exopolysaccharide bound per injured trachea was about 6% of the total used. Hence, there is a clear preference of the exopolysaccharide for injured tissue.

TABLE 2. Effect of various exopolysaccharide concentrations on adherence of mucoid *P. aeruginosa* 2192 to injured mouse trachea

Treatment (μg/ml)[a]	No. of bacteria per 1,000 μm² (mean ± SD)[b]
PBS control	9.0 ± 3.8
10	29.9 ± 9.4
100	27.4 ± 9.3
1,000	35.8 ± 18.8
1,000 (uninjured trachea)	0.6 ± 05

[a] Inocula, 5.2×10^7 CFU/ml.
[b] One of three separate experiments.

TABLE 3. Effect of strain 2192 exopolysaccharide on adherence of various bacterial inocula to acid-injured trachea[a]

Bacterial inoculum (CFU/ml)	No. of bacteria per 1,000 μm² (mean ± SD) with[b]:	
	PBS	Exopolysaccharide
7.4×10^7	52.9 ± 5.0	104 ± 14.9
3.7×10^7	31.8 ± 5.6	91 ± 9.2
1.8×10^7	8.0 ± 4.5	26.7 ± 10.0
7.4×10^6	5.2 ± 2.1	11.4 ± 2.3

[a] Exopolysaccharide concentration, 1,000 μg/ml.
[b] Control versus exopolysaccharide: $P < 0.001$ by Student's t test.

Inhibition of adherence by antibody. We next studied whether the antibody prepared against strain 2192 exopolysaccharide would inhibit adherence of different mucoid strains. The results (Table 4) show that four of the five mucoid strains tested were inhibited from adhering in the presence of antibody. This antibody did not inhibit the adherence of mucoid strain 258 or the nonmucoid revertant NM2192. As noted earlier, this antibody reacted poorly against the exopolysaccharide of strain 258, with a passive hemagglutination assay titer of 1:16 (undiluted). The dilution of antibody used did not cause microscopic or macroscopic agglutination of any of the strains.

DISCUSSION

It has been suggested that the mucoid exopolysaccharide of *P. aeruginosa* is involved in the adherence of this organism (6), but direct proof of this has been lacking. The data presented in the present study provide evidence that the exopolysaccharide may indeed be the adhesin for mucoid strains. Evidence commonly accepted as supporting the classification of a substance or structure as an adhesin includes inhibition of adherence by the purified component, the demonstration that the substance binds to the surface in question, and inhibition of adherence of the whole organism by antibody directed against the substance in question. This study provides supportive data for the latter two lines of evidence, but rather than showing inhibition of adherence, our data show an enhancement of adherence by the mucoid exopolysaccharide. Although this observation does not fit the usual evidence used to characterize an adhesin, it does fit the general definition of how an adhesin should function, i.e., to promote adherence of an organism. We infer that the purified exopolysaccharide in our study functions as a bridge between the exopolysaccharide coat of the organism and the tracheal surface since our second line of evidence demonstrates direct binding to the tracheal surface. This inference means that the polysaccharide ought to bind to bacterial surface, but we were unable to demonstrate this by direct binding experiments (data not shown). We believe that during the spinning and washing of the bacteria to remove excess polysaccharide, this loosely associated substance was easily dislodged.

TABLE 4. Inhibiting effect on binding of mucoid *P. aeruginosa* by of anti-exopolysaccharide antibody[a]

Strain	No. of bacteria per 1,000 μm² (mean ± SD)		P value[b]
	PBS control	Test	
2192	28.6 ± 3.2	5.8 ± 2.0	<0.001
1	7.3 ± 1.1	1.4 ± 1.1	<0.001
M35	14.3 ± 2.3	3.3 ± 1.8	<0.001
OK	24.5 ± 9.2	8.1 ± 2.4	<0.001
258	27.7 ± 8.8	24.4 ± 8.5	NS
NM2192	28.7 ± 7.0	33.1 ± 4.7	NS

[a] Antiserum prepared against strain 2192 and diluted 1/100.
[b] By Student's t test. NS, Not significant.

There is evidence in other systems to support the thesis that exopolysaccharides may serve as adhesins. For example, *Streptococcus mutans* synthesizes glucans which act as adhesins after the initial binding of this organism (7). However, the best evidence in this regard comes from studies of marine organisms (13). One organism of particular interest is *Pseudomonas atlantica*. This organism appears to adhere to glass surfaces by means of an acidic polysaccharide (5). This acidic material is rich in uronic acids (4), resulting in a highly charged macromolecule. In this respect, the material resembles the mucoid exopolysaccharide of *P. aeruginosa*, which is composed of uronic acids and is highly charged (3). Our studies showed an enhancement of adherence by the mucoid exopolysaccharide, but there are no such studies showing enhancement of adherence with *P. atlantica*. However, studies with the algal organism *Chlorella vulgaris* show that different uronic acid-containing materials from various marine sources enhance the adherence of this organism to glass (14). Interestingly, the most active substance was the carbohydrate material from *P. atlantica* T_6C that had a uronic acid-to-neutral sugar molar ratio of 1:1.2. Thus, we see a parallel to our observations on enhancement of binding of bacteria in the presence of exopolysaccharide occurring in nature, with an exopolymer which has similarities to that of *P. aeruginosa*.

This study also supports information on the antigenic composition of different mucoid strains reported by Pier et al. (9). In that study, it was reported that strains 2192 and 1 had similar mannuronic-to-guluronic acid ratios which were different from that of strain 258. It was also reported that antibody against strain 2192 reacted poorly against strain 258, whereas it reacted well with strain 1. Correlates of these data are seen in our adherence assay, in which antibody against strain 2192 exopolysaccharide did not inhibit the adherence of strain 258 but did inhibit the adherence of strains 2192 and 1, as well as that of other strains. It is therefore quite possible that the major antigen in the mucoid exopolysaccharide of strain 2192 is widely represented among mucoid strains. The nonmucoid revertant from strain 2192 was not inhibited by this antibody, although small amounts of the mucoid exopolysaccharide remained on the surface of strain NM2192 (9). This suggests that the nonmucoid revertants do not utilize the exopolysaccharide as an adhesin, a conclusion that is supported by the observation that their adherence is inhibited by pili (11).

The results of this study suggest that antibody against the major epitopes of this exopolysaccharide may function as anti-adhesins. However, since the cystic fibrosis host is unable to clear this organism despite having high levels of antibody against this substance (9), there may be other factors which affect clearance of this organism in addition to inhibition of adherence.

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