INFECTION AND IMMUNITY, May 1981, p. 443-448
0019-9567/81/050443-06$02.00/0

Vol. 32, No. 2

# Influence of Mucoid Coating on Clearance of *Pseudomonas aeruginosa* from Lungs

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Pulmonary infection with mucoid strains of *Pseudomonas aeruginosa* is present in the majority of cystic fibrosis patients with chronic lung disease. It has been postulated that this mucoid coating may act to decrease lung clearance of *Pseudomonas* by limiting access of phagocytes, antibodies, and antibiotics to the bacteria. To determine whether mucoid coating of *Pseudomonas* might decrease intrapulmonary killing, groups of guinea pigs were infected with intrabronchial instillations of equivalent numbers of mucoid and nonmucoid *Pseudomonas*. For this study, mucoid strains of *Pseudomonas* were obtained from cystic fibrosis sputa and passaged on blood agar plates to obtain their nonmucoid revertants. Animals were then sacrificed at timed intervals after infection, and quantitative cultures were performed on lung homogenates. In all cases, mucoid challenge strains retained their mucoid morphology after passage in guinea pig lungs. No difference in killing of mucoid and nonmucoid *Pseudomonas* could be detected at 6, 24, or 48 h after lung infection. Further challenge studies used guinea pigs that were either prevaccinated with lipopolysaccharide *P. aeruginosa* vaccine or else treated with tobramycin sulfate after infection. Nonvaccinated or untreated controls had reduced intrapulmonary killing of *Pseudomonas* compared with vaccinees or treated groups ($P < 0.02$ and $P < 0.01$, respectively). However, there were no differences in pulmonary killing of mucoid and nonmucoid *Pseudomonas* in the presence of either specific antibodies or antibiotic. We conclude from these studies that mucoid coating of *Pseudomonas* does not selectively impede mechanisms of intrapulmonary killing in guinea pig lungs.

Bronchopulmonary disease is now the leading cause of morbidity and mortality among patients with cystic fibrosis (CF) (28). In 60 to 90% of CF patients, chronic and progressive bronchitis is associated with persistent isolation of *Pseudomonas aeruginosa* from sputum cultures (4, 24, 25, 28). The majority of these *Pseudomonas* isolates are distinctive for their production of a copious extracellular polysaccharide material, which has been characterized as an alginic acid (14). These so-called "mucoid" *Pseudomonas* strains are easily identifiable by colonial characteristics and require no specialized media for primary isolation or identification (5, 6, 27). Although mucoid strains account for only 0.8 to 2.1% of clinical *Pseudomonas* isolates in a general population (5), up to 80% of all *Pseudomonas* isolates from CF patients have mucoid characteristics (4, 24, 27).

Once the CF patient harbors mucoid *Pseudomonas* in the lungs, it is virtually impossible to eliminate it with therapy (5, 11, 27). There has been recent speculation that mucoid coating of *Pseudomonas* may offer a survival advantage in the CF respiratory tract by protecting the bacteria from phagocytic cells, antibodies, and even antibiotics (9, 11, 13). Although several in vitro studies have suggested that mucoid coating of *Pseudomonas* may be antiphagocytic (1, 26), this has not been confirmed with in vivo studies. It has been well established that phagocytic cells are the predominant effectors of bactericidal activity in the lungs (8, 29), and a guinea pig model of experimental *P. aeruginosa* pneumonia has recently been described in which studies of intrapulmonary phagocytic cell activity for *Pseudomonas* can be carried out (18–20). Factors which might adversely affect normal pulmonary alveolar macrophage or polymorphonuclear leukocyte functions can be detected in this model by decreased clearance of *Pseudomonas* in the lungs (18, 20). In the present report, we have used this model to explore the hypothesis that mucoid coating of *P. aeruginosa* selectively retards killing of this organism in the lungs.

## MATERIALS AND METHODS

**Animals.** Disease-free Hartley strain guinea pigs (400 g) were obtained from Charles River Laborato-

ries, Wilmington, Mass. Animals were housed in standard cages and fed guinea pig chow (Ralston-Purina Co., St. Louis, Mo.), cabbage, and water.

***P. aeruginosa* strains.** Two different isolates of *P. aeruginosa*, designated strain 1 and strain 3, were used. These strains were obtained from the sputa of two CF patients with chronic *Pseudomonas* bronchitis. Primary isolation and identification were carried out in the Clinical Microbiology Laboratory of Children's Hospital Medical Center, Boston, Mass. After primary isolation from the sputum, both strains were mucoid as defined by others (6, 27). Nonmucoid colonial morphology revertants were obtained for each strain by repeated passages on blood agar plates. All isolates were maintained at −70°C by adding 1 ml of an overnight broth culture of the organisms to a 1-dram (ca. 3.7-ml) glass vial containing 3-mm-diameter glass beads (Curtin Matheson, Woburn, Mass.). Antibiotic sensitivity testing was carried out for the mucoid and nonmucoid revertant of each strain by the standard Kirby-Bauer disk system; serotyping was done and expressed according to the typing scheme of Fisher et al. (7).

For each experiment, a 50-ml bottle of tryptic soy broth was inoculated with two to three glass beads coated with the appropriate organism and incubated at 37°C in a shaking water bath. Organisms were harvested for animal inoculation during declining log phase (20 to 24 h) as determined by growth curves. In selected experiments, organisms were harvested during mid-log growth phase (16 h). Challenge strains to be used for lung infections were washed as previously described (19), and optical densities at 620 nm were determined, using a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Bacterial suspensions were then adjusted to the desired concentrations, using optical densities, to ensure equivalent cell numbers among mucoid and nonmucoid forms of each strain. Exact concentrations were verified by the serial dilution, pour plate technique and expressed as colony-forming units (CFU) per milliliter of final suspension.

**Experimental model of lung infection.** The guinea pig model of *P. aeruginosa* pneumonia used in these studies has been previously described (19). Briefly, 400-g guinea pigs were anesthetized with intraperitoneal pentobarbital, and the trachea was surgically exposed. A 0.5-ml portion of *Pseudomonas*, suspended in isotonic saline, was then directly instilled into the lower respiratory tract via the trachea. The neck was sutured, and animals were positioned upright until awake. It has been shown in this model that survival from infection and efficiency of *Pseudomonas* killing in the lungs are influenced by the size and strain of inocula (19, 20, 22), prior vaccination (19, 22), prior immunosuppression (18, 20), or antibiotic treatment (23). Thus, host defenses as well as bacterial virulence potentials can be altered in this model, and their effects on the pathogenesis of *Pseudomonas* pneumonia may be investigated.

**Influence of mucoid coating on clearance of viable *Pseudomonas* from the lungs.** In each experiment, a comparison was made between in vivo killing of equivalent lung inocula of mucoid and nonmucoid revertant forms of *Pseudomonas*. The occurrence and time of any deaths from experimental pneumonia were also noted. After experimental lung infection, groups of guinea pigs were electively sacrificed with intraperitoneal pentobarbital at timed intervals, lungs were removed aseptically, and both the quantity and colonial morphology of viable *Pseudomonas* in the right lung were determined as previously described (19, 20). Quantities of viable *Pseudomonas* were expressed as CFU × $10^3$ per milliliter of lung homogenate, and differences among study groups were analyzed by the two-tailed Student *t*-test. In selected experiments, left lungs were perfused and fixed with 10% Formalin and prepared for histopathological examination, as described (19, 20).

In further studies, groups of guinea pigs were prevaccinated with a heptavalent lipopolysaccharide *Pseudomonas* vaccine (Pseudogen; Parke, Davis & Co., Detroit, Mich.) (10) and evaluated for the potential of mucoid coating to decrease the in vivo efficiency of opsonic antibodies in the lungs. As in previous studies (19), a 2-week-long series of six injections (50 μg of lipopolysaccharide antigen per kg [body weight] per injection) was used, and serum passive hemagglutinating antibody titers were determined. Groups of guinea pigs vaccinated in this manner were challenged with mucoid or nonmucoid *Pseudomonas* strain 1 (Fisher type 6), and intrapulmonary killing was compared. Nonvaccinated controls were infected and studied simultaneously.

Finally, it has recently been shown that tobramycin sulfate (Nebcin; Eli Lilly & Co., Indianapolis, Ind.) treatment of experimental *Pseudomonas* pneumonia in guinea pigs enhances intrapulmonary killing of *Pseudomonas* (23). To study the potential for mucoid coating to inhibit the in vivo effectiveness of tobramycin, groups of guinea pigs were infected with mucoid or nonmucoid *Pseudomonas* and treated with doses of tobramycin sulfate known to produce effective levels in guinea pigs (1.7 mg per kg [body weight] every 8 h) (23), and intrapulmonary killing was compared among study groups. Again, a group of nontreated controls was also infected and studied simultaneously.

## RESULTS

**Characteristics of *Pseudomonas* challenge strains.** Both *Pseudomonas* strains used in this study were Fisher type 6, and each was sensitive to gentamicin, tobramycin, amikacin, carbenicillin, ticarcillin, and polymyxin B. Results of serotyping and disk sensitivities did not vary between mucoid and nonmucoid revertant forms. Thus, except for colonial morphology, the mucoid and nonmucoid forms of each strain appeared identical.

Accurate comparisons of pulmonary clearance rates of mucoid and nonmucoid *Pseudomonas* depended upon delivery of equivalent inocula to the lungs. To ensure that turbidity from exopolysaccharides in suspensions would not cause artifacts in optical density readings, and to prevent inaccurate CFU determinations due to the described tendency for mucoid *Pseudomonas* to

agglutinate into microcolonies (13), organisms were washed and blended before challenge.

With these precautions, the use of optical densities to provide equivalent inocula for the mucoid versus nonmucoid *Pseudomonas* challenges was reliable. A total of 15 separate comparative challenges were carried out for strain 1. The mean inoculum for the mucoid groups was $2.27 \times 10^8$ CFU (range, 1.4 to 3.3), and for nonmucoid groups the mean was $1.79 \times 10^8$ CFU (range, 1.4 to 2.6). These differences were not statistically significant. Likewise, among five separate challenge experiments using strain 3, the mean inoculum for mucoid groups was $1.5 \times 10^8$ CFU (range, 0.6 to 2.3), and for nonmucoid groups it was $1.1 \times 10^8$ CFU (range, 0.6 to 1.3). Again, differences were not significant. Thus, differences in quantities of viable *Pseudomonas* cultured from the lungs among the study groups should reflect differences in host killing rather than in inoculum sizes.

**Persistence of *Pseudomonas* in the lungs.** Experimentally infected guinea pig lungs were monitored for the persistence of mucoid and nonmucoid *Pseudomonas* to determine the length of time necessary for the host to totally eliminate these organisms from the lungs. Accordingly, pairs of guinea pigs infected with strain 1 mucoid and nonmucoid *Pseudomonas* were sacrificed daily from 1 to 7 days after infection. *Pseudomonas* was isolated on blood agar plates from infected lungs of both groups for the first 3 days after infection, with the mucoid morphological strain being consistently cultured from animals having received this form of the challenge bacteria. The mucoid coating potential, therefore, appeared to be preserved in guinea pig lungs. *Pseudomonas* was not recovered from lungs infected with mucoid strains after the first 72 h of infection; however, occasional nonmucoid *Pseudomonas* colonies were isolated from the nonmucoid-infected animals up to 6 days after infection. Thus, mucoid coating did not appear to favor prolonged persistence in guinea pig lungs.

**Survival and histopathology.** The size of inoculum for both strain 1 and strain 3 was selected to ensure survival among the majority of infected animals. However, occasional deaths occurred during experiments. The number of deaths among mucoid- and nonmucoid-infected groups was tabulated and compared for both challenge strains (Table 1). No increased mortality was found for either strain among the animals infected with mucoid *Pseudomonas* (chi-square analysis). Likewise, microscopic examination of lung sections prepared from animals 24 h after lung challenge demonstrated no difference between mucoid and nonmucoid groups in degree of lung inflammation or lung tissue damage, using previously described criteria (19, 20).

TABLE 1. *Mortality among groups of guinea pigs infected with mucoid and nonmucoid Pseudomonas*

| *Pseudomonas* strain | Mean inoculum size (CFU × $10^8$) | No. of deaths/no. infected | % Mortality |
|---|---|---|---|
| 1 | 1.79 | 8/73 | 11 |
| 1 (mucoid) | 2.27 | 8/65 | 12 |
| 3 | 1.10 | 3/15 | 20 |
| 3 (mucoid) | 1.50 | 2/15 | 13 |

**Pulmonary clearance.** Table 2 compares the quantities of viable *P. aeruginosa* retrieved from the lungs of infected animals. Although more mucoid organisms of strain 1 were present 6 h after infection, this difference was not statistically significant. Similarly, numbers of mucoid and nonmucoid strain 1 *Pseudomonas* isolated 24 and 48 h after infection did not differ significantly. To ensure that strain 1 was not unique in this regard, similar studies were performed on a separate *Pseudomonas* isolate (strain 3). Again, no significant differences in pulmonary killing of mucoid and nonmucoid *Pseudomonas* were detected with this bacterial strain (Table 2). Thus, in the untreated, immunologically intact animals, mucoid coating did not appear to selectively protect *Pseudomonas* from the normal defense mechanisms of phagocytosis and killing.

Since washing of mucoid *Pseudomonas* was carried out before lung challenges, the present analysis was based upon the assumption that mucoid strains were able to produce exopolysaccharide in vivo. Although direct morphological demonstration of mucoid material in vivo was not available in these studies, others have demonstrated this phenomenon (13). It was also reassuring to find that *Pseudomonas* cultured from lungs infected with mucoid strains consistently displayed mucoid colonial morphology. Finally, to infect with mucoid organisms which might direct their metabolic efforts primarily toward exopolysaccharide production rather than maximal mitotic activity, organisms which were entering declining log phase were routinely used for these challenges. To ensure that different results would not occur with organisms harvested at mid-log growth phase, groups of six guinea pigs were challenged with equivalent inocula ($1.6 \times 10^8$ CFU) of strain 1 mucoid or nonmucoid *Pseudomonas* which had been harvested after only 16 h in broth culture. Twenty-four h after lung challenges, the mean CFU per

TABLE 2. *Quantitative cultures of lungs at timed intervals after infection with mucoid and nonmucoid Pseudomonas*

| Time after infection (h) | CFU (× $10^3$)/ml of lung homogenate[a] | | | |
|---|---|---|---|---|
| | Strain 1 | Strain 1 (mucoid) | Strain 3 | Strain 3 (mucoid) |
| 6 | 92.5 ± 18 | 158.0 ± 44 | | |
| 24 | 35.2 ± 11 | 18.8 ± 5 | 5.4 ± 1.5 | 7.6 ± 2.6 |
| 48 | 5.0 ± 1.6 | 4.3 ± 1.5 | 2.2 ± 0.4 | 2.0 ± 0.4 |

[a] Values are expressed as mean ± standard error of mean; six to eight animals per group at each time.

milliliter of lung homogenate was 8.5 ± 3 × $10^3$ for the group infected with nonmucoid *Pseudomonas* and 11.0 ± 3 × $10^3$ for the group infected with mucoid *Pseudomonas*. As before, mucoid- and nonmucoid-infected groups were not significantly different from one another.

It was recently shown that active vaccination of guinea pigs with a lipopolysaccharide *Pseudomonas* vaccine resulted in increased *Pseudomonas*-specific opsonic antibodies in the lungs, which could augment phagocytosis of *Pseudomonas* by alveolar macrophages (21) and increase *Pseudomonas* killing in the lungs (19). To evaluate whether mucoid coating might block the effectiveness of *Pseudomonas*-specific opsonic antibodies in lung tissues, groups of vaccinated animals were challenged with strain 1 mucoid or nonmucoid *Pseudomonas* and compared for intrapulmonary killing. Fourfold or greater elevations in passive hemagglutinating *Pseudomonas* antibody titers were present in the sera of all vaccinated animals (reciprocal, geometric mean titer = 140). The unvaccinated control group contained both mucoid (four)- and nonmucoid (five)-infected animals, and a mean value for *Pseudomonas* in control lungs was determined and compared with a mean value for the mucoid- and nonmucoid-infected vaccinees after 6 h of infection. Vaccinees had significantly fewer ($P < 0.02$, Student's *t*-test) viable *Pseudomonas* in lungs (mean, 58.6 ± 8 × $10^3$ CFU) than controls (mean, 140.9 ± 29 × $10^3$ CFU), confirming previous reports (19, 22). However, when groups of vaccinees were compared for intrapulmonary killing of mucoid versus nonmucoid *Pseudomonas*, no significant difference was seen at 6 h (56.0 ± 10, nonmucoid; 61.2 ± 13, mucoid), 24 h (19.2 ± 7, nonmucoid; 22.4 ± 13, mucoid), or 48 h (17.3 ± 5, nonmucoid; 5.0 ± 1.8, mucoid) after infection (mean values expressed as CFU × $10^3$ per milliliter of lung homogenate, with five animals in each study group). Since increased pulmonary killing of *Pseudomonas* in vaccinated guinea pigs appears to be dependent upon specific opsonic antibodies (21), the present findings suggest that mucoid coating did not block opsonic antibody activity in the lungs.

Likewise, a regimen of tobramycin sulfate, shown earlier to enhance killing of *Pseudomonas* in guinea pig lungs (23), again offered increased killing in lung tissues when treated animals were compared with an untreated control group. As before, the control group included both mucoid- and nonmucoid-infected animals. After 6 h of infection, the mean values (CFU × $10^3$ *Pseudomonas* per milliliter of lung homogenate) were 181.1 ± 35 in controls and 79.0 ± 13 in treated animals ($P < 0.01$, Student's *t*-test). When tobramycin-treated mucoid- and nonmucoid-infected groups were compared, however, there was no evidence that mucoid coating selectively prevented the normal intrapulmonary effectiveness of the antibiotic at 6 h (90.3 ± 20, nonmucoid; 65.8 ± 17, mucoid) or 24 h (25.9 ± 11, nonmucoid; 13.7 ± 6, mucoid) after infection (mean values expressed as CFU × $10^3$ per milliliter of lung homogenate, with six animals in each study group).

## DISCUSSION

The pathogenesis of lung disease in CF is complex and not fully understood (28). It is clear, however, that in a large percentage of CF patients, chronic bronchitis with *P. aeruginosa* is associated with deteriorating lung function (4, 24, 25, 28). A number of clinical observations have been reported which correlate more severe lung disease among CF patients with specific microbiological and immunological settings. These include isolation of the mucoid variety of *P. aeruginosa* from sputum (3, 11, 27), high serum titers of antibodies to *Pseudomonas* exotoxin A and protease (12), elevated gamma globulin levels (17), and increased numbers of *Pseudomonas* precipitins, detected by crossed immunoelectrophoresis (11). In no case, however, has it been determined whether these microbiological or immunological findings are causative in the pathogenesis of CF lung disease, or whether their presence merely reflects the natural consequence of chronic, progressive bronchitis. It has recently been proposed that the peculiar mucoid exopolysaccharide, produced by the majority of *P. aeruginosa* isolated from the

sputa of CF patients, may confer a selective survival advantage in the CF lung by inhibiting normal pulmonary host defense mechanisms (9, 11, 13). The present study has used an animal model, previously shown to be useful in studies of pulmonary host defense (18-23), in an attempt to demonstrate a selective defect in intrapulmonary killing of mucoid-coated *P. aeruginosa*.

A number of in vitro studies have been done to evaluate the influence of mucoid coating of *Pseudomonas* on bacterial susceptibility to phagocytic cells. These studies have produced conflicting data. Schwarzmann and Boring separated slime from a mucoid *Pseudomonas* isolated from the sputum of a CF patient and demonstrated an antiphagocytic property of this material when an excess of slime was mixed with mucoid *Pseudomonas* and polymorphonuclear leukocytes (26). However, unless exogenous slime was added to these leukocyte-bacteria mixtures, no difference in phagocytosis of mucoid and nonmucoid *Pseudomonas* could be demonstrated. Antiserum, raised in rabbits by intravenous injections with a combination of slime and mucoid *Pseudomonas*, was able to reverse the antiphagocytic properties of exogenous *Pseudomonas* slime. Thus, it appeared that slime did not prevent the action of specific opsonic antibodies. In contrast, Baltimore and Mitchell recently reported that certain strains of mucoid *Pseudomonas* isolated from CF patients required greater amounts of antibody for effective opsonophagocytosis than did their nonmucoid revertants (1). Attempts by these investigators to inhibit phagocytosis of nonmucoid *Pseudomonas* by the addition of exogenous slime to bacteria-leukocyte mixtures were unsuccessful, however. Additionally, a preopsonized mucoid *Pseudomonas* stained brightly for the presence of antibody coating when the indirect immunofluorescence technique was used (1). This is in contrast to similar studies by others (16). Finally, it was reported by Govan and Fyfe that mucoid *Pseudomonas* isolates from CF sputa were more resistant to a variety of antibiotics, including tobramycin and carbenicillin (9). Conversely, Thomassen and co-workers recently screened a large number of *P. aeruginosa* isolates from CF sputa for antibiotic susceptibility and found that mucoid strains were more susceptible than nonmucoid strains to tobramycin and carbenicillin (27).

Many of these inconsistencies in the in vitro analysis of mucoid and nonmucoid *Pseudomonas* may relate to the variables of bacterial processing. The amount of mucoid coating will depend upon duration of growth as well as on the harvesting and washing procedures. Clearly, an in vivo system would offer an opportunity to minimize the variables of in vitro analysis and would allow an evaluation of killing of mucoid *Pseudomonas* under more physiological conditions. Although such in vivo analyses are dependent upon the expression of mucoidy in the lung, recent morphological evidence exists to support this phenomenon (13). One limitation in the present analysis was the lack of methodology to directly quantitate and compare exopolysaccharide capsules for mucoid and nonmucoid *Pseudomonas* in the lung tissues. A recently described technique for antibody-mediated stabilization of polysaccharide bacterial capsules (15) may provide a method for such direct morphological comparisons. It should also be noted that *Pseudomonas* grown in specialized media appear capable of producing unusually large amounts of exopolysaccharide (13), and it is possible that organisms grown in such media might be better suited to in vivo studies. Since the original descriptions of mucoid *Pseudomonas* from CF lungs used conventional media, however, and since the biochemical relationship between highly specialized media and CF respiratory fluids is not established, we used conventional culture media for this study.

The difficulty in establishing an animal model to mimic the chronic *Pseudomonas* lung infection seen in CF patients is well known. Cash et al. recently incorporated *P. aeruginosa* into microscopic agar beads and instilled them into rat lungs (2). Nonfatal chronic *Pseudomonas* lung infections were established with this technique. With identical inocula of mucoid and nonmucoid *Pseudomonas* in agar beads, there were no differences in numbers of mucoid or nonmucoid organisms persisting in the lungs up to 35 days after infection. However, the rates of growth and release from agar beads of mucoid and nonmucoid *Pseudomonas* are unknown, and it is uncertain whether valid comparisons of pulmonary clearance rates for mucoid and nonmucoid strains can be made in the presence of such artificial matrix coatings. In the present study, an agar bead matrix was not used so that mucoid coating alone could be evaluated for its potential to reduce killing of *Pseudomonas* in the lungs. The validity of the present analysis was further enhanced by demonstrating that the capacity for mucoid coating of *Pseudomonas* was not lost by in vivo passage in guinea pig lungs. With this in vivo system to monitor leukocyte-bacteria interactions, it did not appear that mucoid coating selectively reduced the effectiveness of intrapulmonary phagocytosis, opsonic antibody activity, or antibiotic therapy, nor did mucoid *Pseudomonas* appear to cause increased mortality among infected animals. The present data thus suggest that the pathogenesis of chronic

lung disease in CF patients with *Pseudomonas* bronchitis involves a more complex series of host-parasite considerations than simply mucoid coating of the bacteria.

## ACKNOWLEDGMENTS

We thank George Cole and Robert Brackett of Warner-Lambert/Parke, Davis, Detroit, Mich., for providing *Pseudomonas* vaccine and assisting with serologies. We also thank Ann McCone, Diana Kuchmy, and Gerald Pier for technical advice and assistance. Finally, we thank Ingeborg Smith for preparation of the manuscript.

This work was supported by Public Health Service grant AI 15411 from the National Institute of Allergy and Infectious Diseases.

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