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Respiratory-Mucin Inhibition of the Opsonophagocytic Killing of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is a frequent respiratory tract colonizer in diseases in which mucociliary clearance is defective. The most striking of these is cystic fibrosis. The reasons for this organism's ability to colonize the respiratory tract and to persist there are not fully understood. Earlier studies showed that *P. aeruginosa* adheres preferentially to tracheobronchial mucin when compared with enterobacteria. We reasoned that if adherence to respiratory mucin protected *P. aeruginosa* from opsonophagocytic killing, then the ability of this organism to chronically colonize the respiratory tract could be partially explained. Using an opsonophagocytic killing assay with human polymorphonuclear leukocytes, we found that respiratory mucin protected six strains of *P. aeruginosa* from opsonophagocytic killing but did not protect poorly adhering strains of *Escherichia coli*, *Staphylococcus aureus*, or group B streptococci. Incubating *P. aeruginosa* with the mucin prior to addition to the opsonic assay inhibited phagocytic killing, whereas incubation of polymorphonuclear leukocytes with mucin did not, suggesting that inhibition was not due to an effect of mucin on leukocytes per se but was a consequence of bacterial adherence to mucin. Further studies indicated no decrease in the binding of either antibody or complement component C3 to the bacterial surface in the presence of mucin. This suggests that phagocytic inhibition may be due to a defect in uptake or destruction of mucin-coated bacteria by the leukocytes. Thus, the adherence of *P. aeruginosa* to respiratory mucin potentially contributes to its persistence in the respiratory tract by interfering with host immune responses.**

Pseudomonas aeruginosa is the predominant respiratory pathogen in patients with cystic fibrosis. The reasons for this predominance are not fully understood. Persistent bacterial colonization of the airways probably involves failure in the local and systemic host defenses. Local defenses consist of the mucociliary apparatus, by which bacteria entrapped in mucus are cleared by ciliary motion. Stagnation of mucus is a feature in cystic fibrosis, and there appears to be a mucociliary clearance defect in this disease (2). Even though the physical clearance system is defective, one would expect the immune response to *P. aeruginosa* to clear this organism from the respiratory tract. This does not occur, and it is reasonable to assume that a functional defect of the immune system allows chronic bacterial colonization. One mechanism may be an acquired immune defect, such as cleavage of immunoglobulins by pseudomonas elastase (7). However, most patients with cystic fibrosis have antibodies to elastase, and their sputum samples do not have pseudomonas elastase activity (5). Another possible acquired immune defect is the cleavage of immunoglobulins and complement components by neutrophil-derived elastase (14). However, such a generalized defect should allow many different bacteria to colonize the airways. Thus, an acquired immune defect must explain the selective maintenance of this organism in the respiratory tracts of cystic fibrosis patients.

Unlike many other gram-negative bacteria which seldom chronically colonize the respiratory tract, *P. aeruginosa* adheres well to respiratory mucin (16). Therefore, adherence to mucin may contribute toward this organism's ability to colonize the respiratory tract. The nature of the survival advantage gained by adherence to respiratory mucin is unclear, since organisms that adhere poorly would also be expected to thrive in stagnant mucus. However, it is possible that binding to mucin could protect *P. aeruginosa* from opsonophagocytic killing and result in a functional immune defect contributing to chronic colonization of the airways in cystic fibrosis. As a corollary, opsonophagocytic killing of nonadherent bacteria would not be inhibited by mucins, and these organisms would be cleared from the respiratory tract.

To test the hypothesis that respiratory mucins interfere specifically with the killing of *P. aeruginosa*, we used an opsonophagocytic bactericidal assay to study the effect of mucin on the phagocytic killing of *P. aeruginosa* as well as poorly adherent strains of *Escherichia coli*, *Staphylococcus aureus*, and group B streptococci.

MATERIALS AND METHODS

Bacteria. Three mucoid *P. aeruginosa* strains—M35, M2192, and M264—used in these and previous studies have been described before (1, 16). Nonmucoid *P. aeruginosa* strains included strains 9882 (lipopolysaccharide [LPS] smooth, Fisher immunotype 2) isolated from the blood of an immunocompromised patient, strain 15921 (LPS smooth, Fisher immunotype 1), a clinical isolate similar to strain 9882, and ATCC 27314 (LPS smooth, Fisher immunotype 3). *E. coli* E739, a blood isolate (4), was obtained from Alan Cross, Walter Reed Army Institute of Medical Research, Washington, D.C. This strain is serum resistant and is phagocytosed only in the presence of 10% fresh human serum. *S. aureus* SA-1 (mucoid) is a mutant of the toxic shock isolate SA-1 and has been described previously (9). Strain M732 of type III group B streptococcus, isolated from the cerebrospinal fluid of a patient with meningitis, has been

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described (6). The gram-negative bacteria were maintained on MacConkey agar plates at room temperature and transferred weekly. The gram-positive bacteria were maintained on tryptic soy agar plates. For use in phagocytic killing assays, the organisms were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C to late log phase, pelleted by centrifugation at 10,000 × *g* for 20 min at 4°C, and suspended to desired concentrations in either minimal essential medium (MEM; Sigma Chemical Co., St. Louis, Mo.) with 1% gelatin, pH 7.4 (MEM-gel), or in RPMI 1640 with 5% heat-inactivated fetal bovine serum (RPMI-FBS).

Opsonins. The following antibodies were used in these studies: rabbit antibody to purified mucoid exopolysaccharide, which reacted with all mucoid *P. aeruginosa* strains (13); rabbit antibody against Fisher immunotypes 1, 2, and 3 LPS, which reacted with strains 15921, 9882, and ATCC 27314, respectively; rabbit antibodies against the purified capsular polysaccharide of *S. aureus* SA-1 (9); and rabbit antibody specific to the type III capsular polysaccharide of group B streptococcus. In all instances, the lowest concentrations of opsonins that yielded maximum bactericidal activity were used. All antisera were heat inactivated at 56°C for 30 min.

Fresh normal human serum obtained from healthy adult volunteers served as a source of complement and was either used immediately or frozen at −70°C and thawed just before use in phagocytosis assays. In some instances, sera were heated at 56°C for 0.5 h to inactivate complement.

PMN. Blood was obtained from healthy adult volunteers by venipuncture after they had given informed consent and was transferred into a conical 50-ml test tube containing 300 U of sodium heparin for anticoagulation. A 20-ml portion of blood was layered on 17 ml of a Ficoll-Hypaque gradient (Mono-Poly Resolving Medium; Flow Laboratories, McLean, Va.) and centrifuged at 1,200 × *g* for 30 min at room temperature. The band enriched for polymorphonuclear leukocytes (PMN) was collected and washed twice with and then suspended in MEM-gel for use in phagocytosis assays. This method yielded >95% pure preparations of PMN. In some instances phagocytic cells were prepared from dextran-sedimented blood as described previously (1). Viability of PMN for the duration of the assays was >99% as tested by the trypan blue dye exclusion test.

Human respiratory mucin. Our method for preparing mucin has been described previously (16). Two different batches were prepared from two different patients at different times. For use in phagocytic killing assays, mucin solutions were prepared in Tris hydrochloride buffer, pH 7.0. Concentrations of mucin used are expressed as a measure of sialic acid content.

Opsonophagocytic killing assays. The opsonophagocytic killing assays used in these studies were modifications of a previously described assay (1). Bacteria were suspended in MEM-gel or in RPMI-FBS and adjusted to the desired concentrations by determining the optical density in a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). The PMN were adjusted to desired concentrations by counting in a hemacytometer. Our preliminary studies showed that opsonophagocytic killing of greater than 50% of the bacterial inoculum occurred at bacteria-PMN ratios of 1:1 to 15:1. In most of our experiments, therefore, ratios of 1:1 to 3:1 were used. The bacteria and PMN were added to test tubes (17 by 100 mm) or sterile microfuge tubes, along with antibody and fresh sera in a final reaction volume of 400 μl. The mucoid strains of *P. aeruginosa* required ca. 0.3% serum, and the nonmucoid strains required 10% serum as a source of complement in addition to antibody for optimal phagocytosis. We also adsorbed the serum serving as the complement source with Formalin-fixed and dried organisms (10 mg/ml of a 1:10 dilution) at 4°C for 30 min if preliminary studies indicated good opsonic killing in the absence of added antiserum. The reaction mixture (25 μl) was added to 225 μl of sterile water, diluted further, and plated to determine the starting inocula at time zero. The mixture was incubated for 1 to 2 h at 37°C with rotation, and 25 μl of the reaction mixture was then diluted with 225 μl of sterile distilled water to lyse the PMN. Serial dilutions were plated on MacConkey agar or tryptic soy agar plates to determine the number of surviving bacteria. From the colony counts, the percentage of the bacterial inocula that had been killed was calculated. Controls included mixtures in which (i) PMN were omitted to determine the survival of bacteria in the presence of antibody and complement, (ii) fresh sera were replaced by heat-inactivated sera to determine whether phagocytosis was dependent on complement, (iii) antibody was omitted to determine whether antibody was required for phagocytosis, and (iv) PMN, sera, and antibody were omitted to test bacterial survival in buffer.

The effect of respiratory mucin on phagocytic killing was determined by adding mucin solutions (100 μl) to the reaction mixtures. To determine whether mucin affected phagocytic killing through an interaction with either bacteria or PMN, bacteria or PMN were first incubated in 300 μl of mucin solutions at 37°C for 30 min and then washed by centrifugation and suspended in 200 μl of buffer before the desired concentrations of antibody, complement, and phagocytic cells or bacteria were added in appropriate volumes of buffer to the phagocytosis mixtures.

Adherence assays. Our method for assessing bacterial adherence to mucin has been described before (16). We had already established that *P. aeruginosa* M35 and M2192 adhered to respiratory mucin (16). We retested the adherence of these strains to respiratory mucin in addition to *P. aeruginosa* 9882, 15921, ATCC 27314, and M264, *E. coli* E739, *S. aureus* SA-1 mucoid, and group B streptococcus M732 in these studies.

Inhibition of binding of antibody and C3 to bacteria by mucin. We used a whole-bacterial-cell enzyme-linked immunosorbent assay (ELISA) (12) to assess whether mucin blocked binding of antibody to cells. Briefly, wells coated with bacteria were incubated with either mucin or medium prior to addition of the rabbit antibodies described above. The remainder of the assay was performed as described (12). The effect of mucin on the binding of C3 to the bacterial surface was tested as follows: 250 μg of purified human C3 (Cytotech, San Diego, Calif.) was labeled with $Na^{125}I$ by using IODO-GEN (Pierce Chemical Co., Rockland, Ill.) as described by the manufacturer. A specific activity of 2.5×10^5 cpm/μg was obtained. A 5-μg amount of labeled C3 was added to a 2% concentration of fresh normal human serum, and 100 μl of this mixture, 100 μl of 1% rabbit antibody to purified mucoid exopolysaccharide or LPS, and either 100 μl of 500 μg of mucin per ml or medium were added to 2×10^8 bacteria. Controls were labeled C3, antibody, and bacteria only. After 1 h at 37°C, bacteria were pelleted, washed twice in phosphate-buffered saline, and then suspended in Protosol (New England Nuclear Corp., Boston, Mass.) and Liquifluor-toluene (New England Nuclear) for scintillation counting. The mean counts of triplicate samples were determined,

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TABLE 1. Adherence of bacteria to respiratory mucin[a]

Mucin batch	Strain	Bacterial inoculum (no. of cells)	Mean no. of adherent CFU/well ± SD[b]
1	*P. aeruginosa* M35[c]	2.9×10^7	20,541 ± 667
	P. aeruginosa M2192[c]	1.1×10^7	21,750 ± 480
	P. aeruginosa 9882[d]	6.0×10^7	46,600 ± 2,114
	E. coli E739	1.1×10^8	917 ± 260
2	*P. aeruginosa* M264[c]	1.1×10^7	2,800 ± 300
	P. aeruginosa 15921[d]	0.8×10^7	58,750 ± 380
	P. aeruginosa ATCC 27314[d]	1.0×10^7	32,000 ± 400
	S. aureus SA-1[c]	2.3×10^7	227 ± 200
	Group B streptococcus M732	3.0×10^7	825 ± 275

[a] Adherence was tested in a previously described microtiter-well method (16).
[b] Mean of at least two experiments done in triplicate.
[c] Mucoid strain.
[d] Nonmucoid strain.

and the binding of C3 to bacteria in the presence and absence of mucin was compared.

Statistics. At least two experiments were done to study each phenomenon tested (n, at least six) and a two-tailed Student's t test was performed to determine whether the differences between controls and test values were statistically significant ($P < 0.05$).

RESULTS

Bacterial adherence assays. Using a previously described microtiter plate assay (16), we studied the adherence of the *P. aeruginosa*, *E. coli*, *S. aureus*, and group B streptococcus strains. Since two batches of mucin were prepared from two different sources, the data are shown that way (Table 1). As before, we found that the *P. aeruginosa* strains adhered more avidly to respiratory mucin than the others. Bacterial adherence to uncoated wells was negligible, as reported in previous studies (data not shown).

Effect of mucin on opsonophagocytic killing of *P. aeruginosa*. Using mucin from the first subject, we found that concentrations of mucin between 2.4 and 3.0 μg of sialic acid equivalents per ml significantly inhibited the phagocytic killing of three *P. aeruginosa* strains but did not inhibit killing of the *E. coli* strain (Fig. 1). Using the second batch of mucin at concentrations of 20 μg of sialic acid equivalents per ml, we found inhibition of killing of three other strains of *P. aeruginosa* but not *S. aureus* or the group B streptococcus (Fig. 1). Bacterial growth was seen when (i) PMN were omitted from the mixtures, (ii) antibody was omitted, or (iii) the serum was heated to inactivate complement. Thus, phagocytic killing was dependent on the presence of PMN, antibody, and complement.

To determine whether the observed inhibition was due to bacteria-mucin interactions or to an inhibitory effect of mucin on PMN, we preincubated either *P. aeruginosa* M35 or PMN with mucin from the first subject, then washed the cells to remove excess mucin, and used the washed bacteria or PMN in phagocytosis assays. The concentration of mucin in these experiments was 5 μg of sialic acid equivalents per ml. We found that opsonophagocytic killing was not inhibited when the PMN were incubated with mucin solutions prior to use. However, opsonophagocytic killing of the bacteria that were incubated with mucin prior to addition to leukocytes and complement was significantly reduced com-

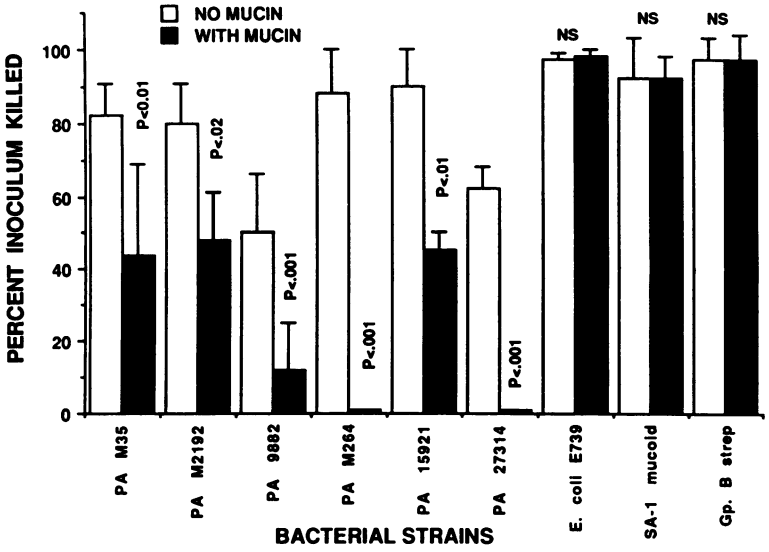

FIG. 1. Inhibition of killing of bacterial strains by human respiratory mucin. The two mucin preparations used with the different strains are as shown in Table 1. The error bar is the standard deviation. P values (Student's t test) compare differences with and without mucin present for each strain. NS, Not significant. PA, *P. aeruginosa*.

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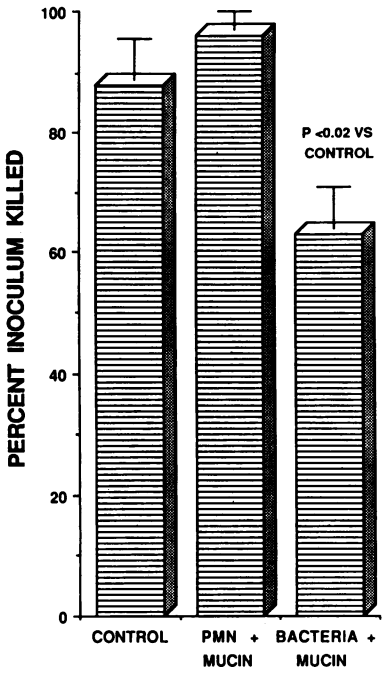

FIG. 2. Inhibition of phagocytic killing of *P. aeruginosa* M35 following incubation of either PMN or bacteria with mucin followed by washing and addition to the rest of the reaction mixture for the phagocytic assay. The error bar is the standard deviation. *P* value determined by Student's *t* test.

pared with controls (Fig. 2); the inhibition observed was less than that seen when bacteria, opsonins, and PMN were all mixed with mucin during opsonic-killing assays with this strain. When the other *P. aeruginosa* strains were incubated with mucin, washed, and then added to PMN, antibody, and complement, inhibition ranged from 55 to 100% of killing seen in the absence of mucin.

Effect of mucin on binding of antibody and C3 to *P. aeruginosa*. Using a whole-bacterial-cell ELISA system, we were unable to document any inhibitory effect of mucin on binding of either mucoid exopolysaccharide-specific antibody to the mucoid strains of *P. aeruginosa* or LPS-specific antibody to nonmucoid strains (Fig. 3). Similarly, we found no inhibitory effect of mucin on binding of [^{125}I]C3 to *P. aeruginosa* strains (Fig. 3).

DISCUSSION

The results reported here show that respiratory mucin inhibited the opsonophagocytic killing of six *P. aeruginosa* strains but did not inhibit the opsonophagocytic killing of strains of *E. coli*, *S. aureus*, and a group B streptococcus. Opsonophagocytic killing of *P. aeruginosa* was not inhibited by incubating leukocytes with mucin prior to use in the assay, whereas incubation of bacteria and mucins inhibited killing. Thus, inhibition was dependent on an initial bacteria-mucin interaction. These findings suggest that adherence of *P. aeruginosa* to respiratory mucin protects them from opsonophagocytic killing, resulting in an acquired immune defect that contributes to the persistence of this organism in the respiratory tracts of patients with defective mucociliary

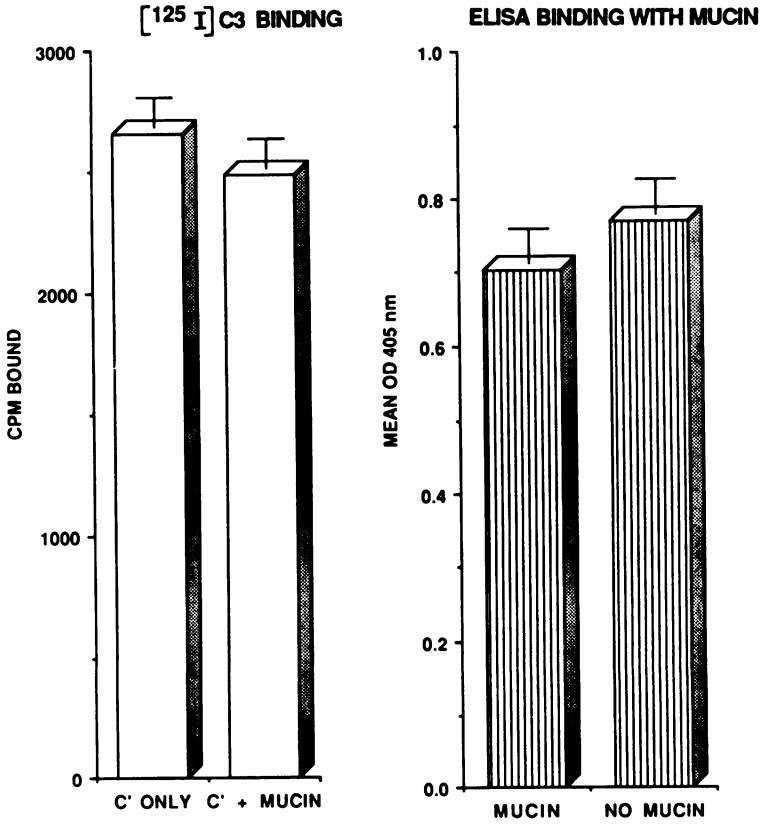

FIG. 3. Inhibition of binding of either [^{125}I]C3 or antibody (ELISA binding) to the six strains of *P. aeruginosa* in the presence or absence of mucins. The error bar is the standard deviation. No significant differences ($P > 0.8$, Student's *t* test) were noted in either assay. C', Complement.

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clearance, i.e., patients with bronchiectasis, posttracheostomy states, and especially cystic fibrosis.

In cystic fibrosis, one puzzling feature has been the ability of *P. aeruginosa* to establish and maintain colonization in the face of seemingly adequate antibody responses, a normal complement system, and adequately functioning phagocytes. On the basis of our findings, we propose that *P. aeruginosa* binds to respiratory mucins present in stagnant mucus secretions, evades opsonophagocytic destruction, and persists in these secretions because the host is unable to clear the organisms by physical and immunological defenses. Extracellular products of bacteria such as exotoxin A and proteases could damage the airways and create further mucus stagnation, resulting in a vicious cycle. These events would allow the heavy colonization of airways and parenchymal lung damage that are seen in cystic fibrosis patients.

The precise mechanisms by which respiratory mucin inhibits the opsonophagocytic killing of *P. aeruginosa* are not yet clear. Other investigators have studied the effect of mucins and sialic acid on the interactions of the immune system with bacteria. Olitzki et al. showed that agglutination of *Salmonella typhosa* by antisera is inhibited by hog gastric mucin (11). More recently, Edwards et al. have shown that sialic acid in the capsule of type III group B streptococci inactivates the alternative pathway of complement (6). Nungester et al. showed that gastric mucin inhibits phagocytic destruction but not phagocytic uptake of *Streptococcus haemolyticus* and *Streptococcus pneumoniae* (10). Finally, Tunnicliff found that gastric and salivary mucin inhibited the phagocytic uptake of *S. albus* and *S. viridans* (15). We found that mucin failed to interfere with either antibody or C3 binding to *P. aeruginosa*, suggesting that it interferes with the interaction of opsonin-coated bacteria and PMN.

Our results and findings from other laboratories suggest that binding to mucins may play a role in determining the bacterial pathogens and flora that exist in specific mucosal sites; e.g., *E. coli*, a common enteric bacterium, adheres to colonic mucus (3). In addition, Kuriyama and Silverblatt have shown that the urinary Tamm-Horsfall glycoprotein inhibits the phagocytosis of type I-fimbriated *E. coli*, a common urinary pathogen (8). These findings suggest that bacteria which adhere to components of mucus secretions at certain mucosal sites are able to colonize these locations, perhaps by binding to mucus components and evading host immune defenses by coating themselves with the host's macromolecules. Bacterial adherence may also provide a similar survival advantage for organisms in nonmucosal sites. Whitnack and Beachey found that *Streptococcus pyogenes* is able to evade opsonophagocytosis by binding to fibrinogen and its degradation products, which abound in inflammatory exudates (17). Thus, adhesin-host receptor interactions may enable bacteria to evade host defenses by (i) providing a means of anchoring to prevent physical clearance, e.g., adherence to cells, and (ii) protecting against phagocytosis, e.g., adherence to mucins, fibrinogen, and Tamm-Horsfall glycoproteins.

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