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Resistance of Mucoid *Pseudomonas aeruginosa* to Nonopsonic Phagocytosis by Alveolar Macrophages In Vitro

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A unique, recently described rat alveolar macrophage cell line (NR8383) was used to study the interaction of the pulmonary immune system with a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa* (SRM-3), its nonmucoid revertant (SRM-3R), and a non-cystic fibrosis isolate (PAO-1). Strain SRM-3 was cultivated in a chemostat system to allow maintenance of an entirely mucoid population. The alveolar macrophage response to the mucoid and nonmucoid strains of *P. aeruginosa* was determined by visually quantitating phagocytosis in acridine orange-stained monolayers and measuring the induction of an oxidative burst as indicated by chemiluminescence and H_2O_2 production. In all experiments, fewer than 2% of the NR8383 cells engulfed the mucoid SRM-3 isolate, while SRM-3R and PAO-1 were phagocytized by 15 and 41%, respectively. Opsonization by normal serum (complement) provided minimal phagocytic enhancement of these strains, whereas specific anti-*P. aeruginosa* antibody slightly elevated phagocytic responses to strains with nonmucoid phenotypes while providing a sevenfold increase in uptake of SRM-3. Chemiluminescent and H_2O_2 responses were comparable with the levels of phagocytosis observed, with very little or no response to the mucoid strain SRM-3. The data indicate that the strains with mucoid phenotypes are refractile to ingestion and that studies which describe ingestion of mucoid strains were likely measuring ingestion of revertants. Alginic acid (2 mg/ml) was found to inhibit stimulation of macrophage response to the opsonized and unopsonized nonmucoid strain PAO-1.

Mucoid *Pseudomonas aeruginosa* is singularly responsible for the morbidity and mortality resulting from the chronic respiratory infection seen in the lungs of cystic fibrosis patients (11, 48). Despite the evocation of an apparently intact immune response (12, 37) and the implementation of a heroic antibiotic regimen, once established this organism is virtually impossible to eradicate. The emergence of the mucoid form of this organism is invariably associated with a poor prognosis for the patient (24, 39) and usually occurs after initial colonization with nonmucoid *P. aeruginosa* as a result of a variety of host selective factors. Protective immunity against *P. aeruginosa* is thought to be achieved through phagocytic killing by peripheral polymorphonuclear neutrophils (PMN) and resident or elicited alveolar macrophages (AM) (1, 12). However, there has been considerable controversy as to the ability of these host cells to ingest and kill mucoid *P. aeruginosa*. Several investigators have demonstrated that mucoid strains are more resistant to phagocytosis than nonmucoid strains are (7, 17, 42). Resistance of mucoid strains to phagocytosis by macrophages and PMN has been attributed to the alginic acid polysaccharide produced by these strains (3, 23, 38, 43), although some investigators have reported no difference between phagocytosis of mucoid and nonmucoid strains (5, 6, 31). The difficulty in assessing the susceptibility of *P. aeruginosa* strains of different phenotypes to phagocytosis is due in part to (i) the rapid reversion of the mucoid phenotype to the nonmucoid phenotype (18, 26) and (ii) in the variability of responses exhibited by freshly derived macrophages or PMN (7).

Phagocytes normally respond to ingested microorganisms by releasing toxic oxygen metabolities, such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals (4, 25). Therefore, the virulence of microorganisms may depend upon either their innate resistance to the reactive products of oxidative metabolism, their production of scavengers which neutralize toxic metabolities, or their ability to induce a metabolic burst, enabling them to evade oxygen-dependent killing by phagocytes. The present study investigated the ability of a continuous alveolar macrophage cell line (22) to phagocytize opsonized and nonopsonized mucoid and nonmucoid strains of *P. aeruginosa* and measured the resulting oxidative burst. Unique to this study were the utilization of a previously described chemostat system for maintaining the mucoid phenotype in vitro (26) and the use of a homogeneous, alveolar macrophage cell system.

(Portions of these studies appeared as a poster presentation at the Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 8 to 13 May 1988.)

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO-1 was obtained from B. Iglewski, Department of Microbiology and Immunology, Oregon Health Sciences University, Portland. The nonmucoid strain DLP and the mucoid strain SRM-3 were isolated from the sputum of cystic fibrosis patients at Santa Rosa Children's Hospital, San Antonio, Tex. To maintain strain SRM-3 in a mucoid form, several colonies were removed from mucoid maintenance agar plates (MacConkey agar base modified by the addition of 50 g of glycerol per liter) (13), suspended in 5% skim milk, and stored at −80°C. The nonmucoid revertant (SRM-3R) was obtained by growth of the mucoid strain in alginate-promoting (AP) medium (described below) at 37°C without aeration for 18 h followed by isolation of the nonmucoid organisms on mucoid maintenance agar plates. An isolated nonmucoid colony was subcultured again and stored as described for the mucoid strain.

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Culture medium. A modification of the AP medium of Mian et al. (32) was used for growth of all strains of *P. aeruginosa*; this has been described in detail elsewhere (26). The AP medium contained 100 mM monosodium glutamate, 100 mM gluconate, 7.5 mM NaH_2PO_4, 16.8 mM K_2HPO_4, and 10 mM $MgSO_4$. The pH was adjusted to 7.0 with 1 M NaOH. The mucoid strain SRM-3 was grown in a chemostat system which allowed constant aeration of the culture at a rate of 0.5 liter/min, a rate needed to maintain an entirely mucoid population of bacterial cells. Nonmucoid strains of *P. aeruginosa* were grown in batch cultures under nonshaking conditions or on mucoid maintenance agar plates.

Exopolysaccharides. Preparation and characterization of the exopolysaccharides of the mucoid strain SRM-3 and the nonmucoid strains PAO-1 and SRM-3R have been previously described (26, 27). The alginic acid polysaccharide in both the bacterial alginate preparation and commercial alginate was quantitated by the harmine assay for uronic acids and pentoses (45) with mannuronic acid as the standard. Lipopolysaccharide was extracted from whole cells by the method of Westphal and Jann (47) for use in enzyme-linked immunosorbent assays.

Macrophages. These studies involved a rat alveolar macrophage NR8383 clone, AgCl1x3A, which was sequentially selected three times from isolated soft agar microcolonies. This clone, which has been previously characterized (22), grows continuously in Ham F12 medium with 15% fetal bovine serum, 100 μg of penicillin per ml, and 100 U of streptomycin per ml.

Phagocytosis assays. The methods of d'Arcy Hart and Young (9) and Pantazis and Kniker (35) were modified to measure the phagocytic response of AM. Sterile 22-mm glass cover slips in petri dishes (35 by 10 mm) were seeded with 0.1 ml of complete medium containing 1×10^4 to 5×10^4 macrophages. After 30 to 60 min of attachment at 37°C, an additional 2 ml of Ham F12 containing 5% fetal bovine serum was added. Cultures were retained in a humidified incubator in 5% CO_2–95% air at 37°C for 24 h before challenge. *P. aeruginosa* cultures containing approximately 10^9 to 10^{10} CFU/ml were spectrophotometrically (at 530 nm) adjusted to 10^8 CFU/ml in Hanks balanced salt solution (HBSS) and further diluted in HBSS to obtain a AM/CFU ratio of 1:300 to 1:500.

Nutrient medium was aspirated from NR8383 cultures, which were then rinsed once with 2 ml of HBSS and inoculated with 1 ml of bacterial suspension. After 1 h at 37°C, inocula were removed and cells were rinsed with 2 ml of HBSS and stained with 6 μg of acridine orange per ml in HBSS for 3 min. After removal of the acridine orange, 2 ml of Karnovsky fixative (M. J. Karnovsky, J. Cell Biol. **27:** 137a, 1965) was added for 2 min; this step served to fix bacteria and cells, remove background acridine orange, and provide a mounting medium. Cover slips were then placed on a microscope slide (cell side down) in residual Karnovsky fluid, and the edges were sealed with Permount (Fisher Scientific Co., Fairlawn, N.J.). The resulting preparations, which were color stable for periods of 25 to 30 min, were analyzed immediately under epifluorescence provided by a 50-W mercury light source (magnification, ×400) (Ortholux; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.). One hundred macrophages per preparation were scored for attachment and internalization of bacteria. Zymosan (Sigma Chemical Co., St. Louis, Mo.) challenges were performed identically to those with bacteria by using boiled, triple-washed zymosan granules (2 mg/ml in 0.85% NaCl) diluted 1:20 in HBSS for an AM/particle ratio of 1:300 to 1:500.

Opsonization of *P. aeruginosa*. Immune rabbit sera were obtained at 30 and 60 days postimmunization from two New Zealand White rabbits immunized intramuscularly with 200 μg of *P. aeruginosa* outer membrane protein prepared from vesicles (19) and emulsified in Freund complete adjuvant. Protein concentration was estimated by a modified Lowry procedure (30). Both rabbits were given a booster immunization (200 μg of protein emulsified in Freund complete adjuvant) approximately 14 days before each bleeding. All sera were stored at −20°C. These antisera had titers of 1:500 to 1:2,000 in enzyme-linked immunosorbent assays when tested against PAO-1, DLP, SRM-3, and SRM-3R immobilized outer membrane preparations. The various bacterial strains were incubated with the antiserum for 30 min at 37°C, followed by centrifugation (4,000 × *g*), washing with HBSS, and suspension in HBSS at a concentration of 10^8 CFU/ml. Terminal blood samplings were taken via cardiac puncture on animals anesthetized with pentobarbital.

H_2O_2 assays. Macrophages (5×10^5 cells) were exposed to stimuli (zymosan or bacterial suspensions) in the presence of homovanillic acid and horseradish peroxidase by using modifications of the methods of Ruch et al. (41). After incubation at 37°C, reactions were terminated by the addition of glycine-NaOH buffer (pH 12), and relative fluorescence was monitored at an emission wavelength of 420 nm and an excitation wavelength of 312 nm on a fluorometer (System 3; Farrand, Valhalla, N.Y.). Alternatively, a Farrand Ratiofluorometer-2 was used with a 7-60 filter in the primary mode and tandem 3-74 and 4-72 filters in the secondary mode. Each assay was extrapolated to standard dilutions of exogenous H_2O_2 under conditions identical to those of the experimental preparations.

CL assays. Chemiluminescence (CL) assays (21, 49) were performed with NR8383 (5×10^5 AM/ml) suspended in HBSS seeded in glass scintillation vials containing luminol (Sigma). Luminol stock (10 mg/ml) was dissolved in dimethyl sulfoxide (American Type Culture Collection, Rockville, Md.) and stored at −20°C. Subsequent dilutions were made in HBSS to a final concentration of 2.5 μg/ml. Stimuli (zymosan or bacterial suspensions) diluted in HBSS were added at time zero, and vials were read immediately and at intervals thereafter. All responses were recorded at ambient temperatures in a scintillation counter (LS150; Beckman Instruments, Inc., Fullerton, Calif.) in "out-of-coincidence" mode through a full window aperture. All studies were done with macrophage preparations which had been allowed to adhere in vials at 37°C for 2 to 3 h, precluding the possibility of spurious CL induced by adherence (R. J. Helmke, V. F. German, and J. A. Mangos, In Vitro Cell Develop. Biol., in press).

Human PMN assays. CL studies with human peripheral blood neutrophils were performed in a different laboratory under slightly different conditions than those described for assays involving NR8383 (V. L. Thomas et al., J. Immunol. Methods, in press). Just before being used in the neutrophil function test, all bacteria (prepared as described above) were pelleted by centrifugation at 5,000 × *g* for 15 min and suspended in phenol red-free HBSS to a concentration of 3×10^8 CFU/ml. Bacteria used in this assay were not opsonized. A 100-μl volume of fresh whole blood was collected in glass capillary tubes, which were then incubated in a humidified chamber at 37°C under a 7% CO_2 atmosphere for 60 min. Clots were removed and the remaining monolayer of phagocytic cells in each capillary tube (approximately 1×10^5 PMN) was rinsed and allowed to equilibrate in buffer for 25 min at 37°C. Each capillary was drained and transferred to

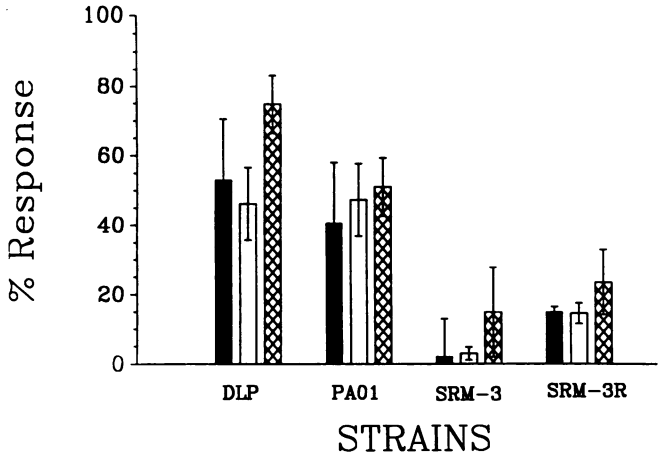

FIG. 1. Phagocytosis of mucoid and nonmucoid strains of *P. aeruginosa*. Percent responses of alveolar macrophages to challenge with unopsonized strains (■), strains opsonized with normal rabbit serum (□), and strains opsonized with anti-*P. aeruginosa* antibody (▨) are shown. One hundred cells per preparation were analyzed for ingestion of one or more bacteria. Data represent two experiments employing quadruplicate preparations.

a cuvette containing 0.5 ml of HBSS, 0.5 ml of the luminol reagent described above, and 0.1 ml of the appropriate bacterial suspension (at a final concentration of approximately 3×10^7 CFU per experimental cuvette). Each cuvette was read for 10 s at 10-min intervals over a 90-min period by using a luminometer. Readings were recorded in relative light units, and each test was performed in triplicate. Phagocytosis of the bacteria by PMN was performed by using the fluorochrome assay described above. Whole blood was allowed to clot on cover slips, and the monolayers of adherent cells were incubated with standardized suspensions of bacteria for 90 min. The monolayers were briefly stained with dilute acridine orange and then examined by fluorescence microscopy.

RESULTS

Phagocytosis of mucoid and nonmucoid strains of *P. aeruginosa* by NR8383. The ability of the alveolar macrophage cloned cell line, NR8383, to phagocytize strains DLP, PAO-1, SRM-3, and SRM-3R was determined (Fig. 1). Unopsonized strains DLP and PAO-1 elicited 53 and 41% responses from NR8383, respectively. The mucoid strain SRM-3 and its nonmucoid revertant SRM-3R, however, elicited very little response from NR8383, with values of 2 and 15%, respectively. Opsonization with normal rabbit serum did not enhance phagocytosis of either the mucoid or the nonmucoid strain, while opsonization with immune sera significantly enhanced phagocytosis of *P. aeruginosa* strains of either phenotype. However, even with the benefit of specific opsonins, mucoid strain SRM-3 was able to elicit only a 15% response from NR8383, and strain SRM-3R was able to elicit only a 24% response. It was empirically but consistently evident that strain SRM-3, along with being relatively insusceptible to phagocytosis, had no tendency to adhere to the surface of the glass cover slips. The nonmucoid and revertant strains, which were subject to engulfment, were consistently observed extracellularly as adherent background organisms (not shown).

CL response of NR8383 to *P. aeruginosa* strains. Actively phagocytizing macrophages emit light or CL, a response which has been shown to be linked to the microbicidal activity of the macrophage. Microbicidal oxygen metabolites such as superoxide and hydrogen peroxide (25) were found to be produced in greater amounts when phagocytes interacted with microorganisms or other suitable stimuli. The sensitivity of the CL assay is markedly enhanced by the addition of luminol, which amplifies the amount of light produced and precludes the need for the cultures or vials in which they are assayed to be dark adapted. The ability of mucoid and nonmucoid strains of *P. aeruginosa* to elicit an oxidative CL response from NR8383 was examined (Table 1). Zymosan was used as a positive control stimulus and produced a maximum CL response of 300×10^4 cpm. Unopsonized strains DLP and PAO-1 elicited lesser responses, with values of 112×10^4 cpm and 117×10^4 cpm, respectively. Unopsonized strains SRM-3 and SRM-3R induced very little response from NR8383, with the nonmucoid revertant eliciting only a slightly greater response than the mucoid parent strain. Opsonization of SRM-3 and SRM-3R resulted in approximately a fourfold increase in oxidative response, but even with opsonization, the mucoid strain SRM-3 triggered very little CL (49×10^4 cpm). The CL response to opsonized PAO-1 and DLP was approximately sixfold greater (288×10^4 and 254×10^4 cpm, respectively) than the response seen with opsonized SRM-3.

TABLE 1. Luminol-enhanced CL by NR8383 1 h after challenge with zymosan and mucoid and nonmucoid strains of *P. aeruginosa*

Stimulus	CL by NR8383 (mean ± SD)[a]	
	Unopsonized	Opsonized (anti-*P. aeruginosa* antibody)
Zymosan	300 ± 43	ND[b]
DLP (nonmucoid)	112 ± 6	254 ± 8
PAO-1 (nonmucoid)	117 ± 46	288 ± 16
SRM-3 (mucoid)	10 ± 2[c]	49 ± 4[c]
SRM-3R (revertant)	46 ± 1	197 ± 8
SRM-3 + zymosan	302 ± 37	ND
Zymosan + SOD[d]	29 ± 3	ND

[a] CL is expressed as 10^4 cpm/5 × 10^5 AM per h; total number of counts was measured at five intervals (0, 5, 15, 30, and 60 min). All data are presented as the means ± standard deviations of triplicate samples from two or three separate experiments.
[b] ND, Not determined.
[c] For mucoid versus nonmucoid strains, $P < 0.001$ (Student's t test), for both opsonized and unopsonized challenges.
[d] Superoxide dismutase, 10 μg/ml; zymosan, 0.1 mg/ml.

In order to determine whether the mucoid strain SRM-3 inhibited the ability of NR8383 to respond to any stimulus, we challenged the NR8383 cells with zymosan and SRM-3 simultaneously. The ability of NR8383 to respond to zymosan with an oxidative burst was not impaired by the presence of SRM-3 (Table 1), indicating that the macrophages were capable of responding to a stimulus but could not significantly bind or respond to the mucoid strain of *P. aeruginosa*. Also shown are data indicating that the CL response was measuring an oxidative product of superoxide, since superoxide dismutase diminished CL by >90%. It should be pointed out that while there is a stoichiometric relationship between O_2 and H_2O_2 produced under identical conditions (4) (Tables 1 and 2), our methods do not permit this extrapolation. The comparative levels of CL versus H_2O_2 production probably reflect the higher sensitivity of the luminol-enhanced assay.

The kinetics of the CL response of NR8383 to mucoid and

TABLE 2. Generation of H_2O_2 in NR8383 2 h postchallenge with zymosan and mucoid and nonmucoid strains of *P. aeruginosa*

Stimulus	H_2O_2 production by NR8383 (mean ± SD)[a]	
	Unopsonized	Opsonized
Zymosan	15.3 ± 3.7	ND[b]
DLP (nonmucoid)	2.7 ± 0.5	9.5 ± 1.3
PAO-1 (nonmucoid)	3.7 ± 0.7	9.7 ± 1.8
SRM-3 (mucoid)	<0.1 ± 0.02[c]	<0.1 ± 0.03[c]
SRM-3R (revertant)	0.2 ± 0.2	1.4 ± 0.3
Zymosan + mannose[d]	12.7 ± 0.2	ND
PAO-1 + mannose	2.6 ± 9.1	8.0 ± 0.6

[a] H_2O_2 generation is expressed as nmol of $H_2O_2/5 \times 10^5$ AM per h. All data are means ± standard deviations of triplicate samples from two separate experiments.
[b] ND, Not determined.
[c] In all cases, the mucoid strain was significantly less stimulatory ($P < 0.01$) than the nonmucoid strains were; comparison of unopsonized mucoid and revertant was not significant.
[d] Mannose (3 mg/ml; Sigma) was added 30 min before stimulus (zymosan, 0.1 mg/ml).

nonmucoid strains of *P. aeruginosa* are shown in Fig. 2. Opsonized PAO-1 elicited a response similar to that of zymosan, both inducing peak responses at 15 min. The response of NR8383 to unopsonized PAO-1 continued to increase during the 60-min period and approached levels equivalent to opsonized PAO-1 only after 60 min. Although NR8383 showed a slight response to opsonized SRM-3 at 5 min, there was essentially no significant response to either opsonized or unopsonized mucoid SRM-3.

Generation of H_2O_2 by NR8383 after challenge with *P. aeruginosa*. Generation of reactive oxygen intermediates by NR8383 was also demonstrated by H_2O_2 production (Table 2). NR8383 was again found to respond similarly to the nonmucoid strains PAO-1 and DLP, with the unopsonized strains resulting in generation of 2.7 and 3.7 nmol, and the opsonized strains stimulating production of 9.5 and 9.7 nmol, respectively. The nonmucoid revertant SRM-3R stimulated production of only 0.2 nmol and only slightly more, 1.4 nmol, when opsonized. The mucoid SRM-3 did not stimulate detectable levels of H_2O_2 by NR8383 even in the presence of opsonins. The addition of 3 mg of mannose per ml prior to stimulus produced little effect on H_2O_2 production in this system.

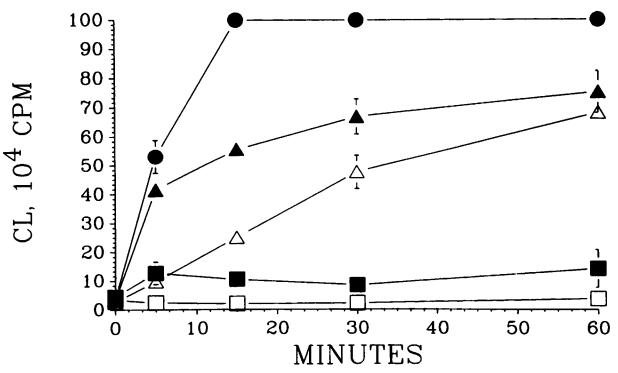

FIG. 2. Kinetics of luminol-enhanced CL response of 5×10^5 alveolar macrophages to zymosan and mucoid and nonmucoid strains of *P. aeruginosa*. ●, Zymosan; △, unopsonized PAO-1; ▲, opsonized PAO-1; □, unopsonized SRM-3; ■, opsonized SRM-3. Data represent the means of triplicate samples in three experiments.

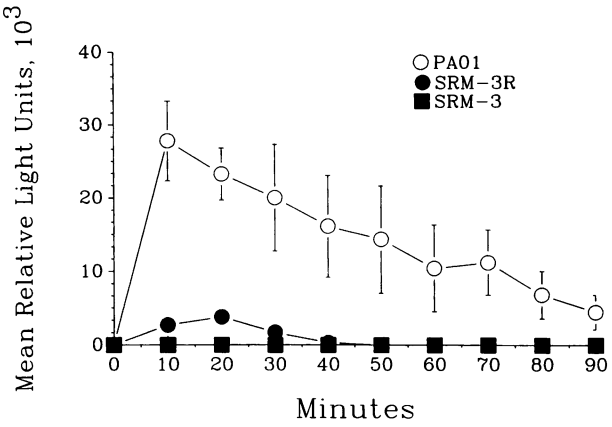

FIG. 3. Luminol-enhanced CL response of human PMN to mucoid and nonmucoid strains of *P. aeruginosa* in the absence of serum opsonins. Two experiments were performed on different days with triplicate samples; PMN were from the same donor.

Phagocytosis of mucoid and nonmucoid strains of *P. aeruginosa* by human neutrophils. In order to demonstrate that the inability of phagocytes to ingest and respond to mucoid SRM-3 was not unique to the alveolar macrophages, we performed phagocytosis and CL assays with human PMN. The induction of CL in PMN after challenge by strains PAO-1, SRM-3R, and SRM-3 was investigated (Fig. 3). Strain PAO-1 readily evoked CL in PMN, with a peak activity of approximately 28,000 mean relative light units. The acridine orange fluorochrome assay revealed 100% of PMN associated with bacteria, and there appeared to be greater than 90% killing of ingested bacteria as evidenced by a well documented (9, 29, 35) orthochromatic color shift from green to red organisms. Strain SRM-3R, with a peak activity of 2,700, was not as effective at stimulating CL by the PMN, even though there was 100% association of PMN with the bacterial cells and greater than 90% killing. It is interesting to note that although the nonmucoid revertant SRM-3R was not as effective as PAO-1 in stimulating CL activity, the rate of uptake between the two organisms, PAO-1 and SRM-3R, was not significantly different as determined by the slope (not shown). Again, strain SRM-3 was not phagocytized by PMN. There was no CL activity, and no SRM-3 bacteria were found to be associated with PMN.

Inhibition of response with alginate. The effect of alginate on H_2O_2 production by NR8383 after stimulation with *P. aeruginosa* PAO-1 was determined by the addition of various concentrations of commercial seaweed alginate to bacteria before challenge (Table 3). In assays with opsonized bacteria, alginate was added to bacterial suspensions before and after opsonization with immune sera. Alginate (2 mg/ml) was also added to zymosan before challenge of NR8383. Alginate at a concentration of 2 mg/ml was found to inhibit H_2O_2 production by NR8383 when challenged with zymosan and unopsonized and opsonized PAO-1. However, the amount of inhibition observed did not correlate with the concentration of alginate during challenge of NR8383 with unopsonized PAO-1. Alginate at a concentration of 2 mg/ml resulted in 24% inhibition of H_2O_2 generation, while 0.5 mg of alginate per ml inhibited production by more than 50%. In assays with opsonized bacteria, H_2O_2 production was substantially inhibited by alginate at concentrations of 0.5 mg/ml or greater, but not in a dose-related fashion.

Alginate purified from strain SRM-3 was also tested to determine its effect on H_2O_2 production by NR8383 upon

TABLE 3. Effect of alginate on H_2O_2 generation by NR8383 after challenge with *P. aeruginosa* PAO-1 or zymosan

Stimulus	H_2O_2 generation by unopsonized NR8383 (mean ± SD)[a]	% Inhibition[b]	H_2O_2 generation by opsonized NR8383 (mean ± SD)[a]	% Inhibition[b]
Zymosan	17.1 ± 6.3		ND[c]	
PAO-1	4.1 ± 0.9		10.6 ± 0.5[e]	
PAO-1 plus[d]:				
2 mg of alginate per ml	3.1 ± 1.1	24.0	3.8 ± 1.1[e]	64.2
1 mg of alginate per ml	2.1 ± 1.0	48.5	4.1 ± 0.5	61.3
0.5 mg of alginate per ml	1.8 ± 0.9	55.9	4.4 ± 0.4	58.5
Zymosan (0.1 mg/ml) plus alginate (2 mg/ml)	8.4 ± 1.2	50.2	ND	

[a] Expressed as nmol of H_2O_2/5 × 10^5 AM per h. All data are presented as means ± standard deviations of triplicates from two separate experiments.
[b] 100% = (nmol H_2O_2 treated/nmol H_2O_2 untreated) × 100.
[c] ND, Not determined.
[d] Indicated concentration of seaweed alginate present during challenge of macrophage with unopsonized or opsonized PAO-1 (1 h, 37°C).
[e] Alginate-treated, opsonized challenges were significantly ($P < 0.001$) inhibited relative to untreated opsonized challenges; unopsonized challenges were not significantly different.

challenge with unopsonized and opsonized PAO-1 (data not shown). The purified bacterial alginate at concentrations of 0.5 and 0.25 mg/ml nonspecifically fluoresced in the standard assay and in a cell-free system. In CL assays, similar concentrations of bacterial alginate inhibited luminol-enhanced CL by NR8383 80 to 95%; cell-free CL was also inhibited.

In order to determine whether the inhibition of an oxidative response to opsonized bacteria in the presence of commercial alginate was due to interference with the opsonization of the bacteria or interference with the response of macrophages to opsonized bacteria, the bacteria were treated with either 2 mg or 0.5 mg of alginate per ml before opsonization, after normal opsonization, or before and after opsonization with immune sera and then used for challenge of NR8383 in the H_2O_2 assay (Table 4). Some inhibition of H_2O_2 response by NR8383 (19.4%) was observed after challenge with bacteria treated with 2 mg of alginate per ml during opsonization, but 0.5 mg of alginate per ml was not inhibitory. The response of NR8383 to opsonized strain PAO-1 was markedly inhibited by 2 and 0.5 mg of alginate per ml, whether it was present only during challenge or during both opsonization and challenge.

DISCUSSION

A comparison of the ability of NR8383 to phagocytize unopsonized mucoid and nonmucoid strains of *P. aeruginosa* revealed significant differences in the susceptibilities of the different strains. While the nonmucoid strains were readily phagocytized, the mucoid strain was not. These results are similar to those recently reported by Cabral et al. (7). Interestingly, the nonmucoid revertant, SRM-3R, elicited only a mild phagocytic response, far below levels seen with the nonmucoid strains DLP and PAO-1. The difference seen in phagocytic response to the unopsonized nonmucoid revertant SRM-3R relative to the nonmucoid strains PAO-1 and DLP may be due to the presence of variable amounts of alginate on the cell surface of the nonmucoid revertant (26), although the involvement of other cell surface components should not be ignored. The phagocytic response by NR8383 to the opsonized mucoid strain was still greatly reduced compared with the response to nonmucoid strains, implying that cell-associated alginate might inhibit opsonic phagocytosis. It has been reported previously, however, that an antibody to alginate from a *P. aeruginosa* strain mediated phagocytic killing by human PMN, suggesting that the antiphagocytic properties of alginate could be overcome by an alginate-specific antibody (1). Previous studies reporting no differences in responses of macrophages to mucoid and nonmucoid strains of *P. aeruginosa* have most likely involved cultures of mixed phenotypes (5, 6, 31).

TABLE 4. Inhibition by alginic acid of H_2O_2 production by NR8383 2 h postchallenge with opsonized *P. aeruginosa* strain PAO-1

Challenge	H_2O_2 production (mean ± SD)[a]	% Inhibition[b]
PAO-1 (untreated control)	6.7 ± 1.5	
Alginic acid during opsonization		
2 mg/ml	5.4 ± 0.6	19.4
0.5 mg/ml	6.7 ± 0.8	0.8
Alginic acid during challenge		
2 mg/ml	3.4 ± 0.8	49.3[c]
0.5 mg/ml	3.3 ± 1.3	50.7[c]
Alginic acid during both opsonization and challenge		
2 mg/ml	3.6 ± 0.8	46.3[c]
0.5 mg/ml	4.4 ± 0.4	34.8[c]

[a] Expressed as nmol of H_2O_2/5 × 10^5 AM per h. All data are expressed as means ± standard deviations of triplicates from three separate experiments.
[b] 100% = (nmol of H_2O_2 treated/nmol of H_2O_2 untreated) × 100.
[c] Significant ($P < 0.5$) relative to untreated control.

It is well known that phagocytosis of microorganisms is accompanied by a dramatic increase in oxygen consumption and the elaboration of reactive oxygen species by the phagocyte (2, 4). Luminol-enhanced CL and hydrogen peroxide assays were therefore used to determine the ability of mucoid and nonmucoid strains of *P. aeruginosa* to elicit an oxidative response from NR8383 and PMN. Luminol-enhanced CL assays, paralleling the phagocytic assays, revealed an inability of NR8383 and PMN to respond to the mucoid phenotype, while the nonmucoid strains elicited marked CL responses. Assays for the detection of H_2O_2 likewise revealed distinct differences between the abilities of mucoid and nonmucoid strains to elicit a response by alveolar macrophages. These results are similar to those of other studies (3, 34), for which it was concluded that the mucoid exopolysaccharide (alginate) alters the surface characteristics of nonopsonized *P. aeruginosa*, rendering them resistant to phagocytosis by PMN and macrophages.

In response to reports that alginate inhibited phagocytosis of nonmucoid *P. aeruginosa* strains (34, 42, 43), we initiated studies in which the nonmucoid strain PAO-1 was treated with extracellular alginate prior to macrophage challenge. Reported physiologic levels of alginate (33, 34) were found to inhibit nonopsonic phagocytosis by NR8383. This was not

surprising, since free sugars have been shown to inhibit binding of a variety of nonopsonized bacteria to phagocytes in vitro (15, 16). This inhibition of phagocytosis by alginate did not occur in a dose-responsive manner, as has been seen previously (34). The addition of a highly charged polysaccharide to these phagocytic systems in which sugars are directly involved in recognition is apparently complex. However, the inhibition of opsonic phagocytosis by alginate did raise the question of whether or not alginate was blocking opsonization of the bacteria or the interaction of opsonized bacteria with the phagocyte. Experiments designed to block either opsonization, phagocytosis of opsonized bacteria, or both indicated that alginate does not interfere with opsonization but does inhibit interaction of the phagocyte with bacteria that would lead to production of H_2O_2. Other reports dealing with the inhibitory effects of alginate on opsonization have revealed similar findings (3, 20).

Results obtained in the CL and H_2O_2 assays using strain PAO-1 treated with purified bacterial alginate from strain SRM-3 suggest some nonspecific interaction of the alginate within the assays. The nonspecific fluorescing in the H_2O_2 assays cannot be explained by us at this time. The inhibition of CL response by bacterial alginate may be due to hypochlorite scavenging by the alginate, which has recently been reported (28). In those studies, seaweed alginate was less effective at scavenging hypochlorite than was bacterial alginate, which may explain why we did not encounter this problem in our studies using commercial alginate. Scavenging of hypochlorite would have no effect on the H_2O_2 assays employed in our studies, since myeloperoxidase is not involved (unpublished observations). It is also unlikely that the lack of macrophage response seen in the H_2O_2 assays in the presence of bacterial alginate is due to contaminating lipopolysaccharide. Lipopolysaccharide could not be detected by assaying for 3-ketodeoxyoctonate by the thiobarbituric acid assay. *Limulus* assays were positive for both seaweed alginate and the bacterial alginate at levels that would have been detectable in the thiobarbituric acid assay. High concentrations of alginate nonspecifically gel in the *Limulus* assay (unpublished observation). It would be of considerable importance to determine the effect of bacterial alginate on the H_2O_2 and CL assay systems. Although it seems unlikely that 0.5 mg of bacterial alginate per ml would be more inhibitory to phagocytosis than 2 mg of seaweed alginate per ml, this possibility cannot be ruled out, since bacterial alginate differs from seaweed alginate in both chemical composition and molecular size (13, 32).

We and other laboratories (7, 14, 29, 36) are intensely interested in the phenomenon of nonopsonic phagocytosis. This capability seems teleologically appropriate to the alveolar macrophage, considering its unique disposition at the environment/mucous interface. Indeed, it has been shown that AM, in contrast to PMN and freshly derived peripheral blood monocytes, can ingest glycoconjugates, such as are contained in many microbial cell walls (and zymosan), via mannose receptors (10, 14, 40). Accordingly, we attempted to demonstrate an inhibitory effect of the zymosan and unopsonized *P. aeruginosa*-induced respiratory burst in NR8383 by D-mannose (Table 2). In contrast to other studies (8, 46), we were unable to demonstrate D-mannose inhibition of H_2O_2 generation.

In contrast to what was shown above, some investigators have reported that PMN are also capable of nonopsonic phagocytosis (7, 8, 29). This is in agreement with our own observations of a CL response to unopsonized *P. aeruginosa* in human PMN (Fig. 2). While we cannot reconcile the fact that PMN have been reported to be devoid of mannose/*N*-acetylglucosamine receptors (44), it is clear that our own PMN preparations, as well as those of Danley and Hilger (8), contained a contaminating population of monocytes, which could provide products of the classical and alternative complement pathways to be deposited locally on otherwise unopsonized particles (14). Still another potential mechanism has been addressed by Lee and co-workers (29), in which adherent organisms were phagocytized and induced CL in the absence of exogenous opsonins. Observations during our own studies consistently revealed a lack of background adherence by the mucoid strain, while the nonmucoid forms, which were ingested by phagocytes, adhered to glass cover slips.

There are probably many bacterial and nonbacterial factors which affect attachment phenomena and interfere with bacterium-phagocyte interactions. Such factors on the surface of *P. aeruginosa* other than alginate could include pili and smooth or rough lipopolysaccharide. Although the chronic lung disease in cystic fibrosis patients involves a complex series of host-parasite interactions, the role of the alginic acid polysaccharide produced by the mucoid strains of *P. aeruginosa* in inhibiting phagocytic response to the bacteria is evident.

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LITERATURE CITED

1. **Ames, P., D. DesJardins, and G. B. Pier.** 1985. Opsonophagocytic killing activity of rabbit antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide. Infect. Immun. **49**:281–285.
2. **Baldridge, C. W., and R. W. Gerard.** 1933. The extra respiration of phagocytosis. Am. J. Physiol. **103**:235–236.
3. **Baltimore, R. S., and M. Mitchell.** 1980. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. J. Infect. Dis. **141**:238–247.
4. **Beaman, L., and B. L. Beaman.** 1984. The role of oxygen and its derivatives in microbial pathogenesis and host defense. Am. Rev. Microbiol. **38**:27–48.
5. **Bender, J. G., A. L. Florman, and D. E. Van Epps.** 1987. Correlation of serum opsonic activity in cystic fibrosis with colonization and disease state: measurement of opsonins to *Pseudomonas aeruginosa* by neutrophil superoxide anion generation. Pediatr. Res. **22**:383–388.
6. **Blackwood, L. L., and J. E. Pennington.** 1981. Influence of mucoid coating on clearance of *Pseudomonas aeruginosa* from lungs. Infect. Immun. **32**:443–448.
7. **Cabral, D. A., B. A. Loh, and D. P. Speert.** 1987. Mucoid *Pseudomonas aeruginosa* resists nonopsonic phagocytosis by human neutrophils and macrophages. Pediatr. Res. **22**:429–431.
8. **Danley, D. L., and A. E. Hilger.** 1981. Stimulation of oxidative metabolism in murine polymorphonuclear leukocytes by unopsonized fungal cells: evidence for a mannose-specific mechanism. J. Immunol. **127**:551–556.
9. **d'Arcy Hart, P. D., and M. R. Young.** 1975. Interference with normal phagosome-lysosome fusion in macrophages, using ingested yeast cells and suramin. Nature (London) **256**:47–49.
10. **DeChatelet, L. R., D. Mullikin, and C. E. McCall.** 1975. The generation of superoxide anion by various types of phagocyte. J. Infect. Dis. **131**:443–446.
11. **di Sant'Agnese, P. A., and P. B. Davis.** 1976. Research in cystic fibrosis. N. Engl. J. Med. **295**:481–485.
12. **Doggett, R. G., and G. M. Harrison.** 1972. *Pseudomonas*

aeruginosa: immune status in patients with cystic fibrosis. Infect. Immun. **6**:628–635.
13. **Evans, L. R., and A. Linker.** 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. **116**:915–924.
14. **Ezekowitz, R. A. B., R. B. Sim, G. G. MacPherson, and S. Gordon.** 1985. Interaction of human monocytes, macrophages and polymorphonuclear leukocytes with zymosan in vitro. J. Clin. Invest. **76**:2368–2376.
15. **Freimer, N. B., H. M. Ogmundsdottir, C. C. Blackwell, I. W. Sutherland, L. Grahame, and D. M. Weir.** 1978. The role of cell wall carbohydrates in binding of microorganisms to mouse peritoneal exudate macrophages. Acta Pathol. Microbiol. Scand. Sect. B **86**:53–57.
16. **Glass, E., J. Stewart, and D. M. Weir.** 1981. Presence of bacterial binding "lectin-like" receptors on phagocytes. Immunology **44**:529–534.
17. **Gosciniak, G., J. Maresz-Babezyszyn, and K. Grzybek-Hryncewica.** 1984. Phagocytosis and intracellular killing of mucoid and nonmucoid variants of *Pseudomonas aeruginosa* by polymorphonuclear leukocytes: effects of specific immune sera. Arch. Immunol. Ther. Exp. **32**:467–479.
18. **Govan, J. R. W.** 1975. Mucoid strains of *Pseudomonas aeruginosa*: the influence of culture medium on the stability of mucus production. J. Med. Microbiol. **8**:513–522.
19. **Hancock, R. E., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
20. **Hann, S., and D. S. Holsclaw.** 1976. Interactions of *Pseudomonas aeruginosa* with immunoglobulins and complement in sputum. Infect. Immun. **14**:114–117.
21. **Hatch, G. E., A. Spock, D. E. Gardner, and D. B. Menzel.** 1980. Differences between particulate and peptide stimuli on activation of oxidant production in alveolar macrophages. Chest **77**: 267–269.
22. **Helmke, R. J., R. L. Boyd, V. F. German, and J. A. Mangos.** 1987. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. In Vitro Cell Develop. Biol. **23**:567–574.
23. **Hoiby, N.** 1974. *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta Pathol. Microbiol. Scand. Sect. B **82**:551–558.
24. **Hoiby, N., V. Andersen, and G. Bendixen.** 1975. *Pseudomonas aeruginosa* infection in cystic fibrosis. Acta Pathol. Microbiol. Scand. Sect. C **83**:459–468.
25. **Iyer, G. Y. N., M. F. Islam, and J. H. Quaslel.** 1961. Biochemical aspects of phagocytosis. Nature (London) **192**:535–541.
26. **Krieg, D. P., J. A. Bass, and S. J. Mattingly.** 1986. Aeration selects for mucoid phenotype of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **24**:986–990.
27. **Krieg, D. P., J. A. Bass, and S. J. Mattingly.** 1988. Phosphorylcholine stimulates capsule formation of phosphate-limited mucoid *Pseudomonas aeruginosa*. Infect. Immun. **56**:864–873.
28. **Learn, D. B., E. P. Brestel, and S. Seetharama.** 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. Infect. Immun. **55**:1813–1818.
29. **Lee, D. A., J. R. Hoidal, C. C. Clawson, P. G. Quie, and P. K. Peterson.** 1983. Phagocytosis by polymorphonuclear leukocytes of *Staphylococcus aureus* and *Pseudomonas aeruginosa* adherent to plastic, agar or glass. J. Immunol. Methods **63**:103–114.
30. **Markwell, M. A. K., S. M. Haas, L. L. Biebeo, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein in samples. Anal. Biochem. **87**:206–210.
31. **Meshulan, T., H. A. Verbrugh, and J. Verhoef.** 1982. Opsonization and phagocytosis of mucoid and nonmucoid *Pseudomonas aeruginosa* strains. Eur. J. Clin. Microbiol. **1**:112–117.
32. **Mian, F. A., T. R. Jarman, and R. C. Righelato.** 1978. Biosynthesis of exopolysaccharide by *Pseudomonas aeruginosa*. J. Bacteriol. **134**:418–422.
33. **Ohman, D. E., and A. M. Chakrabarty.** 1982. Utilization of human respiratory secretions by mucoid *Pseudomonas aeruginosa* of cystic fibrosis origin. Infect. Immun. **37**:662–669.
34. **Oliver, A. M., and D. M. Weir.** 1983. Inhibition of bacterial binding to mouse macrophages by Pseudomonas alginate. J. Clin. Lab. Immunol. **10**:221–224.
35. **Pantazis, C. G., and W. T. Kniker.** 1979. Assessment of blood leukocyte microbial killing by using a new fluorochrome microassay. RES J. Reticuloendothel. Soc. **26**:155–170.
36. **Parod, R. J., and J. D. Brain.** 1986. Immune opsonin-independent phagocytosis by pulmonary macrophages. J. Immunol. **136**:2041–2047.
37. **Pennington, J. E., H. Y. Reynolds, R. E. Wood, R. A. Robinson, and A. S. Levine.** 1975. Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. **58**:629–636.
38. **Reynolds, H. Y., J. A. Kazmierowski, and H. H. Newball.** 1975. Specificity of opsonic antibodies to enhance phagocytosis of *Pseudomonas aeruginosa* by human alveolar macrophages. J. Clin. Invest. **56**:376–385.
39. **Rivera, M., and M. B. Nicotra.** 1982. *Pseudomonas aeruginosa* mucoid strain. Its significance in adult chest diseases. Am. Rev. Respir. Dis. **126**:883–886.
40. **Roos, D., A. A. M. Bot, M. L. J. Van Schaik, M. DeBoer, and M. R. Daha.** 1981. Interaction between human neutrophils and zymosan particles: the role of opsonins and divalent cations. J. Immunol. **126**:433–440.
41. **Ruch, W., P. H. Cooper, and M. Baggiolini.** 1983. Assay of H_2O_2 production by macrophages and neutrophils with homovanillic acid and horseradish peroxidase. J. Immunol. Methods **63**:347–357.
42. **Ruhen, R. W., P. G. Holt, and J. M. Papadimitriou.** 1980. Antiphagocytic effect of PSA exopolysaccharide. J. Clin. Pathol. **33**:1221–1222.
43. **Schwarzmann, S., and J. R. Boring III.** 1971. Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. Infect. Immun. **3**:762–767.
44. **Stahl, P., P. H. Schlesinger, E. Sigardson, J. S. Rodmon, and Y. C. Lee.** 1980. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell **19**:207–215.
45. **Wardi, A., H. William, S. Allen, and R. Varma.** 1974. A simple method for the detection and quantitative determination of hexuronic acids and pentoses. Anal. Biochem. **57**:268–273.
46. **Warr, G. A.** 1980. A macrophage receptor for (mannose/glucosamine) glycoproteins of potential importance in phagocytic activity. Biochem. Biophys. Res. Commun. **93**:737–745.
47. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. **5**:83–91.
48. **Wood, R. E., T. F. Boat, and C. F. Doershuk.** 1976. Cystic fibrosis. Am. Rev. Respir. Dis. **113**:833–878.
49. **Yanai, M., and P. C. Quie.** 1981. Chemiluminescence by polymorphonuclear leukocytes adhering to surfaces. Infect. Immun. **32**:1181–1186.