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Nonopsonic Phagocytosis of Strains of *Pseudomonas aeruginosa* from Cystic Fibrosis Patients

DAVID P. SPEERT,[1,2]* FERESHTEH EFTEKHAR,[1] AND MARTIN L. PUTERMAN[1,3]

Division of Infectious Diseases, Department of Pediatrics,[1] Department of Microbiology,[2] and Faculty of Commerce,[3] University of British Columbia, Vancouver, British Columbia, Canada V6H 3V4

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Pseudomonas aeruginosa is the predominant respiratory pathogen in patients with cystic fibrosis, but its mechanism of persisting in pulmonary secretions is poorly understood. We observed that three nonmucoid cystic fibrosis *P. aeruginosa* strains were phagocytized and one strain resisted phagocytosis by human polymorphonuclear leukocytes in the absence of serum. Phagocytosis was assessed by luminol-enhanced chemiluminescence, inspection of stained smears, bactericidal assay, reduction of nitroblue tetrazolium dye, and electron microscopy. Phagocytosis, determined by visual inspection, occurred at 35°C but not at 4°C. Nonopsonic phagocytosis was inhibited most efficiently by D-mannose, mannose-containing saccharides, and D-fructose. Opsonin-dependent phagocytosis of *P. aeruginosa* and of zymosan was not markedly inhibited by mannose, suggesting different leukocyte receptors for nonopsonic and opsonic phagocytosis.

Pseudomonas aeruginosa has lately emerged as an important pathogen in individuals with impairment of host defenses due to cystic fibrosis, thermal injury, and malignant tumors (21, 25). Other investigators have defined the opsonic requirements for phagocytosis and killing of *P. aeruginosa* by human polymorphonuclear leukocytes (PMN) (2, 16, 28) and by macrophages (20) and have attempted to explain why strains from patients with cystic fibrosis resist phagocytosis (1, 23). In studies with cystic fibrosis *P. aeruginosa* isolates, we observed that phagocytosis occurred in the absence of serum and was inhibited by certain sugars. Observations on the nonopsonic phagocytosis of *P. aeruginosa* by human PMN form the basis of this report.

MATERIALS AND METHODS

Bacterial strains. Mucoid strains of *P. aeruginosa* were cultured from the sputum of patients with cystic fibrosis and identified by oxidase reaction, growth at 42°C, pigment production, and the API 20E system (Analytab Products, Inc., Plainville, N.Y.). They were serotyped by using the Difco typing system (Difco Laboratories, Detroit, Mich.). Strain P-1 (type 9/10) was from the University of Minnesota Hospitals, Minneapolis. Strains C-1 (type 6/9/10), C-46 (type 9/10), and C-96 (nontypable) were from British Columbia's Children's Hospital, Vancouver. Nonmucoid spontaneous laboratory revertants were frozen at −70°C in Mueller-Hinton broth with 8% dimethyl sulfoxide and used as seeds for each experiment. For all experiments, nonmucoid revertant bacteria were grown on Mueller-Hinton agar at 35°C for 18 h, removed with a sterile swab, washed thrice in phosphate-buffered saline (pH 7.4), and adjusted spectrophotometrically (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.) to 10^9 bacteria per ml in Hanks balanced salt solution (HBSS) with 0.1% gelatin (GHBSS). All four strains were very sensitive to the bactericidal effect of pooled normal human serum; >99% killing occurred after incubation at 35°C for 60 min in 5% serum.

Serum. Venous blood was drawn from five healthy adults and allowed to clot for 30 min at room temperature. The serum was decanted after centrifugation, pooled, and frozen in aliquots at −70°C for use in separate experiments.

PMN. Heparinized venous human blood was layered on mono-poly resolving medium (Flow Laboratories, Inc., McLean, Va.) and centrifuged at 300 x *g* for 30 min. The PMN layer was carefully pipetted off, washed twice in GHBSS, and adjusted by hemocytometer to contain 10^6 PMN per ml. Approximately 96% of the cells obtained were PMN. Greater than 98% were viable as assessed by trypan blue dye exclusion.

Sugars. D-Mannose, α-methyl-D-mannoside (αMM), mannan, α-methyl-D-galactoside, D-glucose, and D-fructose were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Solutions (10%) were prepared in GHBSS, filter sterilized, kept at 4°C, and diluted before each experiment.

Zymosan preparation. The method of Mills et al. (13) was followed for zymosan preparation. Zymosan (Sigma) was boiled for 1 h in HBSS (10 mg/ml), centrifuged, cooled, resuspended in phosphate-buffered saline, and opsonized (1 part in 3 parts 100% pooled human serum) for 15 min. It was again centrifuged and resuspended in HBSS.

Luminol-enhanced CL. For luminol-enhanced chemiluminescence (CL) a modification of the method of Mills et al. (13) was used. Glass scintillation vials were dark adapted and then filled with 4.4 ml of GHBSS; to some, one or another of the above sugars was added. To this was added 10^{-7} M luminol (5-amino-2,3 dihydro-1,4-phthalazinedione [Sigma]) dissolved in dimethyl sulfoxide and further diluted in phosphate-buffered saline and 0.1 ml of a suspension of 10^9 bacteria per ml. The vials were further dark adapted. Under red light illumination, 1 ml of 10^5 PMN per ml was added to each vial, which was then agitated manually for 20 s. Immediately thereafter, CL was measured at ambient temperature for 20 s in a Beckman LS-6800 liquid scintillation counter in which the photomultiplier tubes had been adjusted out of coincidence. CL was measured every 12 min in each of 22 vials, and the cycle was repeated 10 times. All samples were analyzed in duplicate, and the mean CL value was computed. In some experiments 10^{-6} M luminol and 5 mg of opsonized zymosan were added to the vials. All experiments were repeated on at least 3 separate days with PMN from different donors. Peak CL varied substantially

* Corresponding author.

from day to day, but the differences among experimental conditions were reproducible. Results of typical experiments are reported.

Bactericidal assay. For the bactericidal assay, a modification of the method of Quie et al. (18) was used in which serum was omitted from the phagocytosis mixture. To polypropylene snap-cap tubes (12 by 75 mm; BD Labware, Oxnard, Calif.) were added 10^6 PMN and 10^6 bacteria with or without one of several sugars. Tubes were rotated end over end at 35°C, and samples were removed at 0, 30, and 120 min and diluted in ice-cold water to lyse PMN. Serial dilutions were made and viable bacteria were counted by the spread-plate technique. In some tubes GHBSS was substituted for PMN and some were held stationary. Using this method with normal PMN, there is consistently >90% killing of *Staphylococcus aureus* 502A and serum-resistant *P. aeruginosa* strain PAO1 in 30 min in the presence of 8% serum and no killing in the absence of serum (D. P. Speert, Y. Kim, and R. Grandy, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no 349, 1981).

Visual assessment of phagocytosis. To polypropylene tubes (12 by 75 mm; BD Labware) were added 10^7 bacteria and 10^5 PMN with or without one of several sugars. The tubes were rotated end over end for 60 min at 35°C (or at 4°C in one set of experiments) and then centrifuged at 26 × *g* for 10 min. The cells were gently washed twice more, resuspended in 0.5 ml of GHBSS, and deposited on a glass slide, using a Cytospin 2 cytocentrifuge (Shandon Southern Products Ltd., Astmoor, England) at 600 rpm for 5 min. The slides were air dried, heat fixed, and stained with crystal violet. The identity of each slide was unknown to the observer at the time of the bacterial count, and the number of bacteria within the cytoplasm of each of 50 cells was quantitated. Although absolute counts varied from day to day, relationships among experimental conditions were reproducible.

NBT reduction. A 0.2-ml portion of a PMN suspension (10^6/ml) was placed on a clean glass cover slip, incubated at 35°C for 30 min, and rinsed with phosphate-buffered saline. A drop of bacterial suspension (10^8/ml) or GHBSS was placed on the cover slip over the PMN and incubated at 35°C for 60 min with gentle rotation. Some cover slips were first coated with *Escherichia coli* endotoxin (Associates of Cape Cod, Woods Hole, Mass.); PMN were then added. Cover slips were washed with saline and then placed, PMN side down, on a drop of nitroblue tetrazolium dye (NBT) medium (Sigma) on a clean glass slide and incubated for 20 min at 35°C. The cover slips were then gently removed, air dried, fixed in methanol for 1 min, rinsed in distilled water, air dried, stained with basic fuchsin for 30 sec, rinsed with distilled water, air dried, and mounted on a glass microscope slide. A total of 400 PMN were examined from each slide. Positive cells were those with blue material within their cytoplasm. Negative cells had pink nuclei and no cytoplasmic staining.

Electron microscopy. The procedure of Root et al. (22) for electron microscopy was followed. A total of 10^8 bacteria and 10^7 PMN were combined in a polypropylene vial and rotated end over end for 60 min. A 1-ml amount of glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) was added, the mixture was centrifuged at 150 × *g* for 15 min, and the supernatant was decanted. Glutaraldehyde was again added for 15 min, and the pellet was washed in sodium cacodylate. The pellet was then postfixed in 1% osmium tetraoxide, embedded in agar, dehydrated in a graded series of alcohols, and embedded in Epon 812. It was finally sectioned in 60-nm-thick slices and poststained with uranyl acetate and lead. Samples were viewed with a Phillips 400 electron microscope operating at 80 kV.

TABLE 1. Influence of various saccharides on phagocytosis of nonopsonizied *P. aeruginosa* strain P-1

Saccharide[a]	Avg bacteria/PMN[b]	Avg square root of bacteria/PMN[c]
Control (no saccharide)	14.08	3.64
α-Methyl-D-galactoside	13.21	3.51
D-Fucose	12.41	3.44
D-Glucose	10.59	3.08
D-Mannose	9.04	2.82
αMM	7.98	2.62
D-Fructose	7.66	2.63
Mannan	1.10	0.76

[a] All saccharides were at 1% (wt/vol).

[b] Averages are from replications of the experiment on 2 separate days.

[c] The standard error for tests of pairwise difference is 0.13. Pairwise differences of the square root exceeding 0.40 are significant at the 0.05 level.

Statistics. Data in Tables 1 to 4 were analyzed by analysis of variance. The dependent variable was taken to be the square root of the count of bacteria/PMN. This transformation was used so that statistical inference based on the normal distribution would be valid (3). Differences were considered significant if the simultaneous *P*-value based on Bonferroni's method (3) for all tests in a particular experiment was <0.05. Computations were performed with BMDP computer programs P1D, P7D, and P2V.

RESULTS

P. aeruginosa strain P-1 was well phagocytized by human PMN in the absence of serum as assessed by visual inspection (Table 1) and by induction of CL (Fig. 1). Phagocytosis was significantly inhibited ($P < 0.0018$) when mannan, D-fructose, αMM, D-mannose, or D-glucose was present in the phagocytosis mixture (Table 1). Phagocytosis was not markedly inhibited by D-fucose or α-methyl-D-galactoside as assessed by visual inspection (Table 1). α-Methyl-D-galactoside and D-glucose inhibited CL, but to a lesser degree than the other sugars. CL was inhibited to a greater extent by α-methyl-D-galactoside than was phagocytosis, as assessed by visual inspection. The inhibitory effect was reversible; it was not seen if either bacteria or PMN were preincubated with αMM, washed, and then added to the phagocytosis system (data not shown). There was a dose-related inhibition of CL by αMM (Fig. 2). Phagocytosis occurred far more efficiently at 35 than at 4°C ($P < 0.0001$) in both the presence and the absence of αMM (Table 2). When PMN from a patient with chronic granulomatous disease were incubated with strain P-1, no CL was produced.

Mannose-inhibitable nonopsonic phagocytosis was also seen with *P. aeruginosa* strains C-46 and C-96 (Table 3; Fig.

TABLE 2. Influence of incubation temperature and presence of αMM on phagocytosis of nonopsonized *P. aeruginosa* strain P-1

Temp (°C)	Avg bacteria/PMN[a]	
	Control (no saccharide)	1% αMM
4	0.59	0.61
35	6.61	2.05

[a] Averages are from replications of the experiment on 2 separate days.

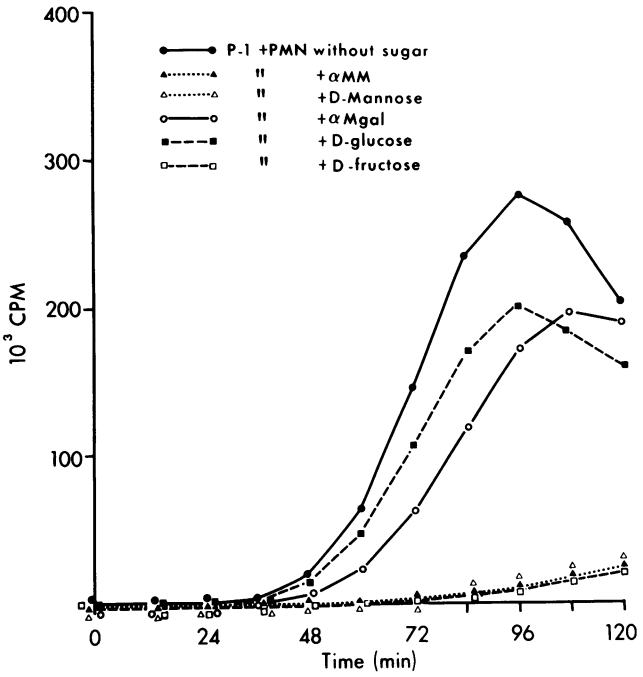

FIG. 1. Effect of various sugars on the neutrophil CL response induced by *P. aeruginosa* strain P-1 in the absence of serum opsonins. αMgal, α-methyl-D-galactoside. All sugars were at 1% (wt/vol).

3). As with strain P-1, there was inhibition by both D-glucose and αMM ($P < 0.0001$), but αMM was more inhibitory than D-glucose for strains P-1, C-46, and C-96 ($P < 0.0001$). Strain C-1 was poorly phagocytized by PMN in the absence of serum as assessed by visual inspection (Table 3) and CL (Fig. 3).

Further experiments were conducted to determine whether mannose-containing saccharides interfered with opsonin-dependent phagocytosis. All four *P. aeruginosa* strains were killed by normal human serum and in its presence did not induce a CL response with PMN. Therefore, neither CL nor an opsonin-dependent phagocytic killing assay could be used. Nonetheless, by visual inspection of stained smears (Table 4), it was determined that αMM significantly inhibited nonopsonic phagocytosis ($P < 0.0001$) but did not interfere with opsonic uptake ($P = 0.13$). αMM and D-glucose did not totally interfere with CL by quenching (Fig. 4). Opsonized zymosan induced a CL response in the presence or absence of the sugars. There was a slightly lower peak CL in the presence of the sugars, but αMM was less inhibitory than D-glucose. αMM inhibited CL with nonopsonized *P. aeruginosa* more profoundly than with opsonized zymosan. It appeared that separate leukocyte receptors were involved in opsonic and nonopsonic uptake of *P. aeruginosa* by human PMN.

TABLE 3. Effect of saccharides on nonopsonic phagocytosis of various strains of *P. aeruginosa*

Bacterial strain	Avg bacteria/PMN[a]		
	Control (no saccharide)	1% αMM	1% D-Glucose
C-96	16.62	5.48	14.70
C-46	11.98	5.34	8.38
P-1	6.74	2.34	5.90
C-1	0.94	0.42	0.08

[a] Numbers are from a representative experiment.

TABLE 4. Effect of αMM on phagocytosis of opsonized and nonopsonized *P. aeruginosa* strain P-1

Condition	Avg bacteria/PMN[a]	
	Not opsonized	Opsonized
Control (no saccharide)	7.62	9.02
1% αMM	2.54	7.70

[a] Numbers are from a representative experiment.

CFU were counted to determine whether *P. aeruginosa* cells were killed by PMN in the absence of serum opsonins (Table 5). There was an approximately 10-fold decrease in viable counts of strain P-1 in the presence of PMN but essentially no loss of viability of strain C-1. When mannan and αMM were present, the bactericidal effect of PMN was diminished. Mannan and αMM did not cause bacterial clumping as no decrease in CFU was seen in their presence.

To confirm that an oxidative metabolic burst occurred with nonopsonic phagocytosis, NBT reduction was assessed in PMN exposed to the four *P. aeruginosa* strains. The three strains that were well phagocytized non-opsonically (P-1, C-46, C-96) induced reduction of NBT but strain C-1, which resisted phagocytosis, did not (Table 6).

Particulate stimuli can induce an oxidative metabolic burst in PMN by simple surface contact without ingestion (9). To document that the *P. aeruginosa* cells were in fact being phagocytized rather than simply perturbing the PMN membrane, we utilized electron microscopy. Multiple sections were examined and bacteria were seen within phagocytic vacuoles in many of the PMN (Fig. 5).

DISCUSSION

Serum opsonins (complement and immunoglobulin) are generally required for phagocytosis and killing of bacteria by PMN (10). Nonetheless, some bacterial species, including *E. coli* (11, 12, 24), *Neisseria gonorrhoeae* (19), and *Klebsiella pneumoniae* (17), are susceptible to nonopsonic phagocytosis. To this list certain strains of *P. aeruginosa* should now be added.

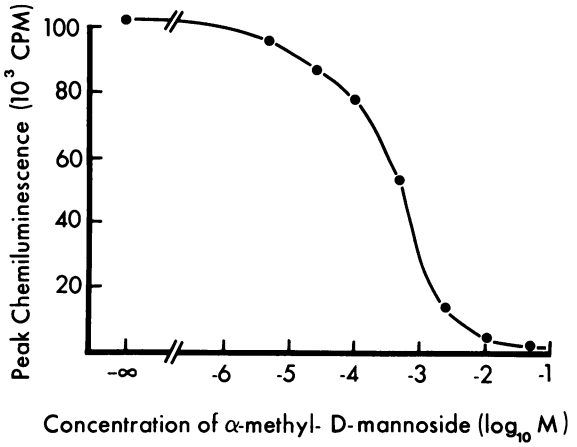

FIG. 2. Dose effect of αMM on opsonin-independent CL stimulated by *P. aeruginosa* strain P-1 ($-\infty$ corresponds to zero concentration).

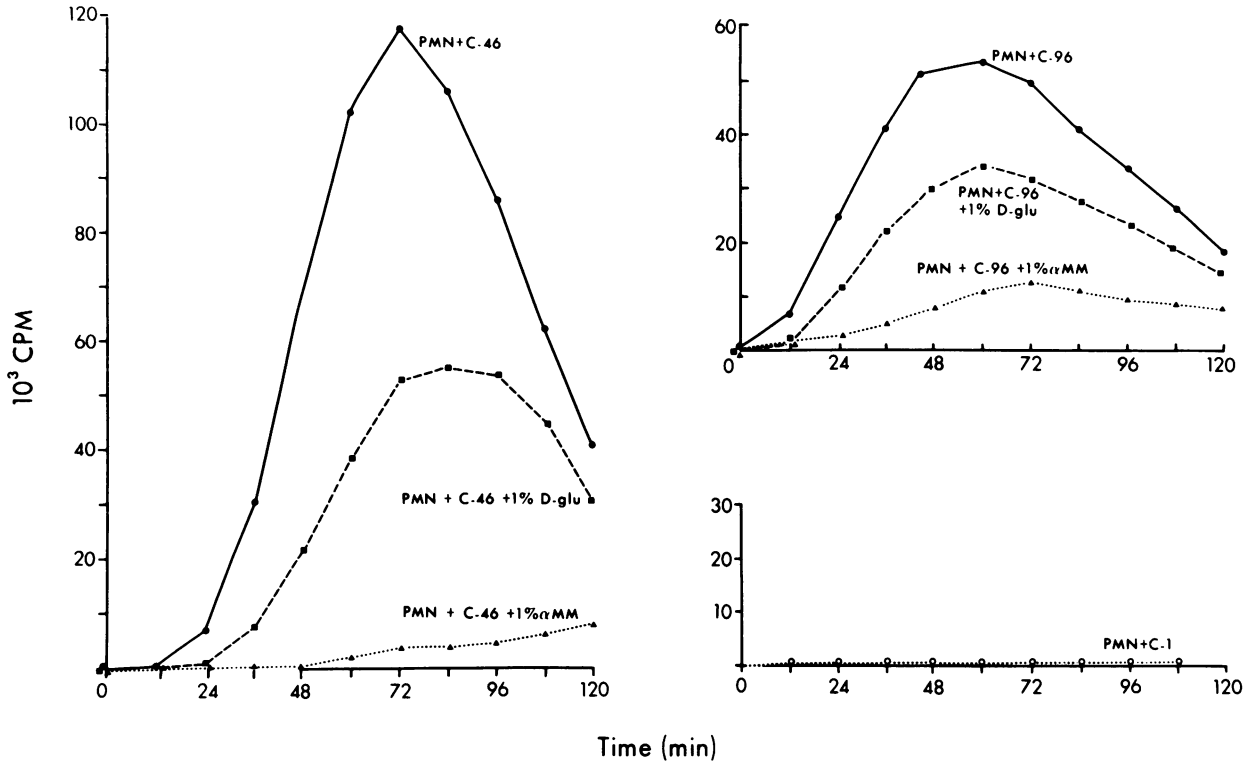

FIG. 3. CL response of human neutrophils with three *P. aeruginosa* strains in the presence and absence of sugars. D-glu, D-Glucose.

The mechanism for nonopsonic phagocytosis of *E. coli* has been well described (15, 24). There are obvious similarities between *P. aeruginosa* and *E. coli* in their nonopsonic phagocytosis by PMN. Only certain strains are phagocytized, but those that are ingested induce a CL response (12).

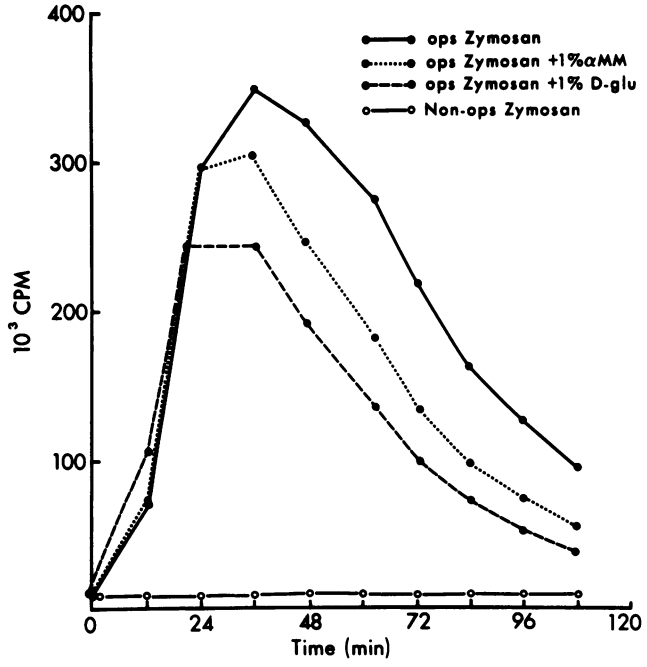

FIG. 4. Effect of sugars on neutrophil CL response induced by zymosan opsonized with normal human serum. D-glu, D-Glucose.

The uptake of both species is inhibited by mannose and mannose-containing saccharides (11, 12, 15). Nonopsonic phagocytosis and adherence of *E. coli* to epithelial cells is mediated by pili (15). The mechanism by which *P. aeruginosa* cells are recognized by PMN remains to be determined, although it has been shown that adherence to epithelial cells is mediated by pili (27).

A question that remains unanswered in these studies is the mechanism by which pseudomonas adhere to, and are phagocytized by, PMN. *P. aeruginosa* cells are piliated (4, 5) and, as already stated, these pili mediate attachment to epithelial cells (27). As opposed to *E. coli*, *P. aeruginosa* will only adhere to trypsinized epithelial cells from which fibronectin has been removed. Adherence can be blocked with purified pili, antiserum to the homologous strain, and killing with heat or Formalin (26). As far as we know, the effects of specific saccharides on adherence of *P. aeruginosa* to epithelial cells have not been investigated.

TABLE 5. Nonopsonic killing of *P. aeruginosa* strains P-1 and C-1 by human PMN in the presence or absence of saccharides

Condition			CFU/ml at time:		% Reduction, in viability
Strain	Saccharide	PMN[a]	0	120 min	
P-1	None	+	8.0×10^5	1.1×10^5	86.5
P-1	None	−	7.6×10^5	6.5×10^5	14.5
P-1	1% αMM	+	8.4×10^5	2.7×10^5	68.3
P-1	1% αMM	−	7.9×10^5	7.6×10^5	3.8
P-1	1% Mannan	+	1.2×10^6	1.1×10^6	8.3
P-1	1% Mannan	−	6.9×10^5	1.2×10^6	0
C-1	None	+	5.6×10^5	4.4×10^5	21

[a] +, PMN present; −, PMN absent.

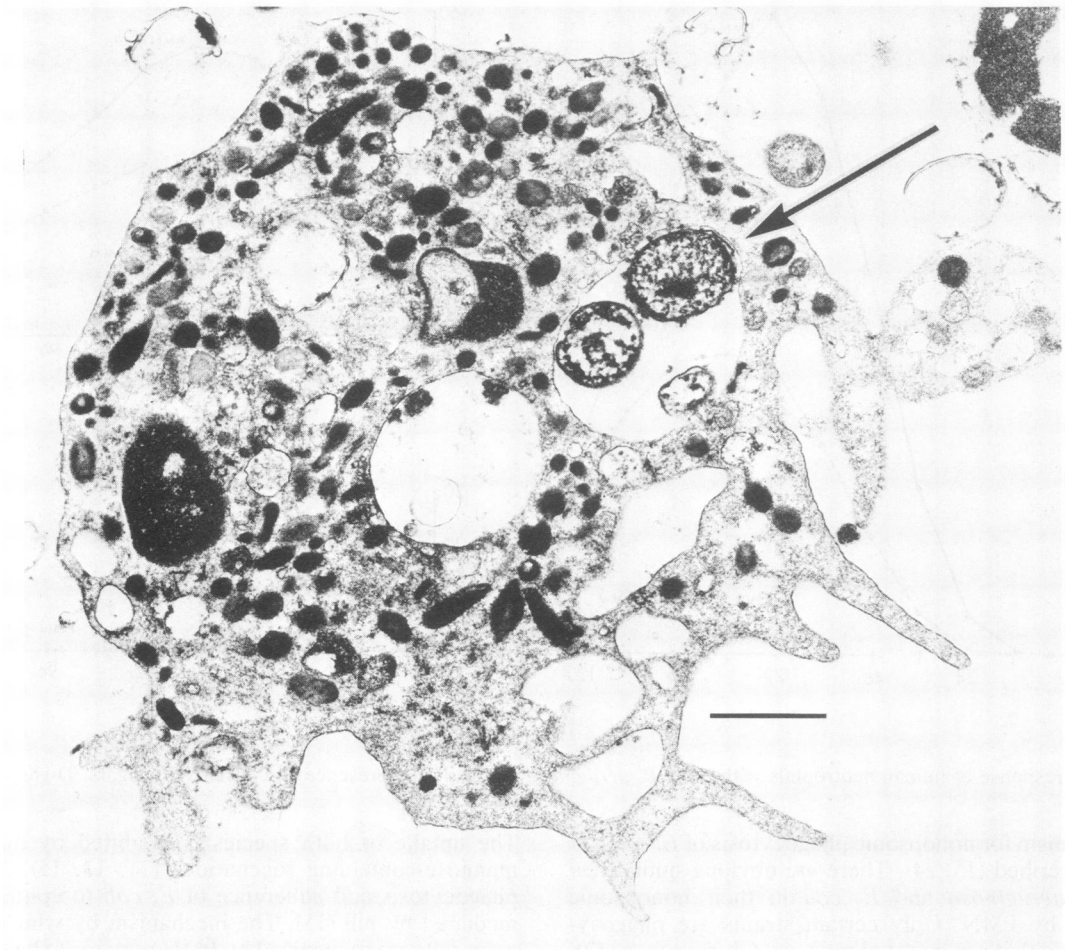

FIG. 5. Electron micrograph of a human neutrophil that has phagocytized *P. aeruginosa* strain P-1 in the absence of serum. Arrow indicates two bacteria within a phagocytic vacuole. Bar, 1 μm.

Both *E. coli* and *P. aeruginosa* produce lectins capable of agglutinating erythrocytes. The *E. coli* lectin is mannose sensitive, is thought to be a component of pili, and mediates attachment to epithelial and phagocytic cells (15). *P. aeruginosa* lectins agglutinate human erythrocytes that have been pretreated with neuraminidase or papain (6). Two different lectins are produced by pseudomonas, one (PAI) that binds to D-galactose specifically (7) and a second (PAII) that binds to L-fucose, D-mannose, L-galactose, and D-fructose (8). Further studies are currently under way in our laboratory to characterize the bacterial ligand involved in nonopsonic recognition of *P. aeruginosa* by human PMN.

TABLE 6. Effect of nonopsonized *P. aeruginosa* on reduction of NBT by human PMN

Condition	% of neutrophils reducing NBT
PMN + P-1	96.8
PMN + C-96	70.4
PMN + C-1	19.8
PMN + *E. coli* endotoxin	95.9
PMN + HBSS	16.9

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