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Nonopsonic Phagocytosis of *Pseudomonas aeruginosa* by Macrophages and Polymorphonuclear Leukocytes Requires the Presence of the Bacterial Flagellum

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Whereas the mechanism of nonopsonic phagocytosis of *Pseudomonas aeruginosa* has been described, the bacterial ligands required are poorly understood. To identify the requisite bacterial ligands, studies with isogenic mutants of *P. aeruginosa* PAK lacking pili, flagella, and the RpoN sigma factor were undertaken. The RpoN mutant, lacking pili, flagella, and nonpilus adhesins, bound poorly and was resistant to ingestion by both macrophages and neutrophils. Pili were not absolutely required for binding or phagocytosis of *P. aeruginosa*. The presence of a flagellum was not required for binding of *P. aeruginosa* to macrophages but was critical for the subsequent internalization of the bacterium, suggesting that this factor or a surface ligand associated with its assembly was responsible for stimulation of nonopsonic phagocytosis.

Pseudomonas aeruginosa is the predominant cause of lung infection in patients with cystic fibrosis (CF). Several *P. aeruginosa* virulence factors that may contribute to pathogenesis during initial colonization of the respiratory tract and subsequent persistence during chronic infection have been characterized (10). However, the specific factors that may interfere with the phagocytic clearance of *P. aeruginosa* from the CF lung remain poorly understood. The initial interaction of pulmonary phagocytic cells and infecting bacteria probably occurs in the absence of serum opsonins, and work in this laboratory has focused on the mechanism of nonopsonic phagocytosis of *P. aeruginosa* (4, 11–13). Once infection has been established, the inflammatory process creates an environment which likely interferes with the clearance of *P. aeruginosa*. Cleavage of complement and phagocytic receptors by bacterial and inflammatory proteases may lead to a receptor-ligand mismatch, reducing the efficiency of opsonophagocytosis (14), and subsequent growth of *P. aeruginosa* as mucoid microcolonies may enable it to evade phagocytic clearance (1). Despite the presence of opsonizing antibody in patients with CF, *P. aeruginosa* converts to a serum-sensitive phenotype and is able to persist in the lung during chronic colonization, suggesting that opsonophagocytic clearance and complement-mediated lysis are defective (10).

Recently, we demonstrated that *P. aeruginosa* isolates collected from chronically colonized CF patients are frequently nonmotile; they lack the surface factors flagella, pili, and nonpilus adhesins which are under the control of the alternative sigma factor, RpoN (4). These studies showed that wild-type CF isolates, lacking a flagellum, are resistant to nonopsonic phagocytosis, while those lacking pili but retaining their motility are susceptible to ingestion (4). *P. aeruginosa* CF isolates lacking both a flagellum and pili and having phenotypes similar to those of *P. aeruginosa* RpoN mutants (15) are completely resistant to ingestion. We suggested that the loss of these surface features during chronic colonization of patients with CF may be a mechanism of bacterial persistence (4). The current study was undertaken to formally assess the role of each of these RpoN-dependent surface factors in nonopsonic phagocytosis. Although previous studies have assessed the role of *P. aeruginosa* pili, flagella, and nonpilus adhesins in susceptibility to phagocytosis by macrophages (3, 5) not one of these studies (i) was able to differentiate between bound and ingested bacteria, (ii) used mutants that were all derived from the same parental isolate, (iii) facilitated contact between nonmotile bacteria and macrophages by centrifugation, or (iv) examined the nonopsonic phagocytosis by neutrophils of these *P. aeruginosa* isogenic mutants. Using methods developed in this laboratory, we have extended previous studies and examined the role of individual surface ligands in binding and ingestion of *P. aeruginosa* by phagocytes in the absence of serum opsonins.

Interaction of *P. aeruginosa* PAK isogenic mutants with murine macrophages. To determine which bacterial surface factors mediate binding by macrophages in the absence of serum opsonins, *P. aeruginosa* PAK and its isogenic mutants were tested in a nonopsonic mouse macrophage ingestion assay (4, 12). The isogenic mutants of *P. aeruginosa* PAK used in this study were obtained from Stephen Lory, University of Washington, Seattle, and their genotypic and phenotypic features are summarized in Table 1. *P. aeruginosa* P1, a nonmucoid derivative of a mucoid CF strain (Table 1), was included in all phagocytic assays as a control. Macrophages were harvested from the peritoneal cavities of female BALB/c mice 3 days after elicitation by thioglycolate injection and prepared for phagocytic assay exactly as previously described (4). *P. aeruginosa* (approximately 2×10^7 viable bacteria) was added to adherent macrophages (10^5; an infection ratio of 200 bacteria per macrophage) and incubated for 1 h at 37°C. The monolayers were then washed with phosphate-buffered saline (PBS), fixed with methanol, and stained with Giemsa, and the number of bacteria associated with each macrophage was determined by light microscopy (4). The mean ± standard error of the mean (SEM) and Student's *t* test for independent means were used to assess differences in the interaction with phagocytes of

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TABLE 1. Features of *P. aeruginosa* PAK isogenic mutants and *P. aeruginosa* P1

Strain	Relevant phenotype or genotype	Abbreviated name	Growth in static broth	Reference or source
PAK-SR	Spontaneous Str^r mutant of PAK	PAK	+	2
PAK-NP	PAK-SR, *pilA*::*tet*	Pil$^-$ mutant	+	8
PAK-MS591	PAK-SR, *fliC*::*gent*	Fla$^-$ mutant	−	S. Lory
PAK-NI	PAK-SR, *rpoN*::*tet*	RpoN$^-$ mutant	−	2
P1	Motile, flagellated, sparsely piliated	P1	+	4, 13

P. aeruginosa PAK and its isogenic mutants. To facilitate bacterium-to-macrophage contact of nonmotile *P. aeruginosa*, bacteria were centrifuged (2,000 × *g* for 10 min) onto the macrophage monolayers prior to the ingestion assay.

The levels of binding of the PAK mutants under normal and centrifuged conditions are shown in Fig. 1A. Both the *P. aeruginosa* P1 control and the PAK parental strain bound well to macrophages under normal conditions; forced contact increased the binding of strain P1 marginally and did not alter the binding of strain PAK (Fig. 1A). Under normal conditions, all the isogenic mutants bound to macrophages significantly less than the parental strain. The absence of surface pili reduced the binding of PAK by approximately 55%, and the lack of the flagellum reduced binding of strain PAK by 84%. Centrifugation of the pilin and flagellin mutants onto the macrophage monolayers increased binding to approximately 80% of that of the parental strain. The RpoN mutant, lacking flagella, pili, and nonpilus adhesins, associated very poorly with macrophages, and binding was only slightly increased by centrifugation (Fig. 1A). Binding of *P. aeruginosa* PAK to macrophage monolayers in the absence of opsonins appeared to require motility for optimal binding and may occur via pilus and nonpilus adhesins.

Ingestion of strain PAK and its isogenic mutants by murine macrophages is shown in Fig. 1B. Extracellular bacteria were lysed with lysozyme, permitting differentiation between intracellular and adherent bacteria (12). Strain PAK was ingested substantially less than strain P1. All the isogenic mutants of strain PAK were phagocytosed significantly less than the parental isolate under both normal and centrifuged conditions (Fig. 1B). Ingestion of strain PAK was dependent on the presence of pili; the pilin mutant was ingested at 30% of the control rate (Fig. 1B). The flagellin mutant was resistant to nonopsonic ingestion; phagocytosis was only slightly increased by forced contact with the monolayer. The RpoN mutant was absolutely resistant to nonopsonic ingestion, and this resistance was not overcome with centrifugation of the bacteria onto the macrophage monolayer. Ingestion of *P. aeruginosa* PAK by macrophages in the absence of serum opsonins, although heavily dependent on the presence of pili, appeared to also require both the bacterial flagellum and other RpoN-dependent factors.

Because the mutants used in this study were constructed by insertion of antibiotic resistance cassettes into structural genes encoding the RpoN-dependent surface factors (Table 1), complementation with the wild-type gene was carried out for the flagellin and the RpoN mutants in order to assess whether restoration of phagocytic potential was possible. Murine mac-

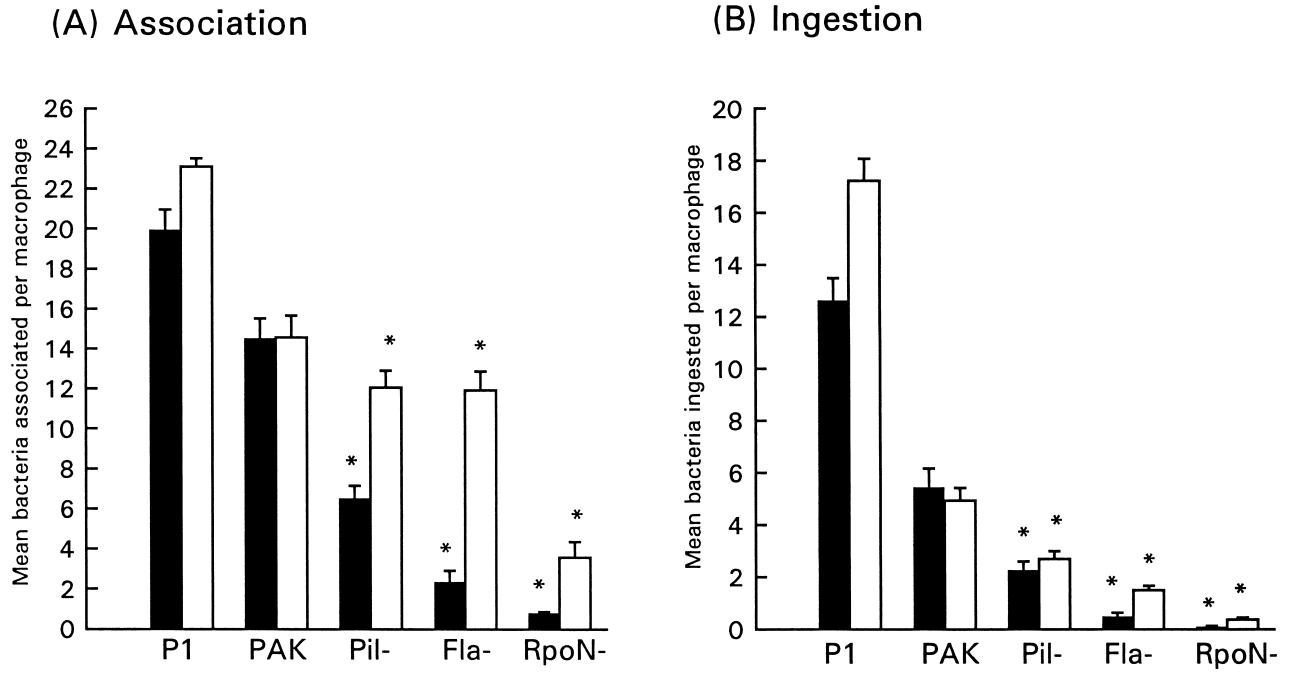

FIG. 1. Murine macrophage phagocytosis of *P. aeruginosa* P1 and PAK and its isogenic mutants. Phagocytosis under normal assay conditions and after centrifugation is shown by the filled and open bars, respectively. Error bars indicate the SEM ($n = 6$ experiments), and the asterisk indicates significant difference compared with the value for the PAK control ($P \leq 0.05$).

TABLE 2. Murine macrophage phagocytosis of complemented PAK flagellin and RpoN mutants

Strain and complementing plasmid (reference)	Complemented gene or feature	Phagocytosis (no. of ingested bacteria)[a]
PAK-SR	Parental strain	8.00 ± 0.46
PAK-MS591	Flagellin mutant	0.01 ± 0.005
pPT244 (16)	*fliC*	3.9 ± 0.15
pDN18 (16)	Vector control	0.06 ± 0.06
PAK-NI	RpoN mutant	0.01 ± 0.003
pPT212 (15)	*rpoN*	1.22 ± 0.07
pSP329G (15)	Vector control	0.03 ± 0.01

[a] Values are means ± SEM; $n = 3$.

rophage phagocytosis of the complemented mutants of strain PAK is shown in Table 2. The phagocytic assays were carried out without centrifugally forcing bacteria onto the macrophage monolayers. Mobilization of plasmid pPT244, carrying the *fliC* gene (16), into the flagellin mutant restored expression of the flagellum and also increased the phagocytic susceptibility of this mutant to approximately 50% that of wild-type PAK (Table 2) (the vector control remained resistant to nonopsonic ingestion). Complementation of the RpoN mutant with plasmid pPT212, encoding the *rpoN* gene (15), only partially restored the phenotype of PAK-NI, with few motile bacteria visible by light microscopy (data not shown). The degree of phagocytosis of the complemented PAK RpoN mutant was only slightly increased and remained well below that of the wild-type PAK (Table 2) (PAK-NI carrying the vector alone was resistant to phagocytosis). Partial complementation of phenotype has been previously observed with "RpoN-mutant-like" isolates from CF infection (4) and also with *P. aeruginosa* PAK-NI (3a) and may result from the complex nature of regulatory events associated with sigma factor.

Absence of RpoN-dependent factors renders *P. aeruginosa* resistant to nonopsonic ingestion by human phagocytes. The interaction of *P. aeruginosa* isogenic mutants lacking RpoN-dependent surface factors with human phagocytic cells has not been examined. Therefore, evaluation of nonopsonic phagocytosis of *P. aeruginosa* PAK and its isogenic mutants was also carried out with human monocyte-derived macrophages (Mϕ) and neutrophils (PMNs) to provide a comparison with the data obtained with murine macrophages.

Monocytes, harvested from the heparinized venous blood of healthy adult volunteers, were cultured in Teflon beakers for 6 days as described previously (13). Approximately 5×10^4 Mϕ were used in each phagocytic assay, and phagocytosis was performed as described previously (13). Bacterial ingestion was assessed as described above for murine macrophages, except that the lysozyme-water wash was omitted (because it detached the human macrophages from the monolayer); all cell-associated bacteria were therefore counted. Because fewer Mϕ were used than in the murine phagocytic assay, the infection ratio of bacteria to macrophages was considerably higher, approximately 400 to 1. Human Mϕ appeared to interact with the same *P. aeruginosa* ligands as the murine macrophages (Fig. 2A). As with the murine macrophages, the $RpoN^-$ and Fla^- strains associated with human Mϕ less than the parental strain (Fig. 2A), indicating the importance of motility in the adhesion process (the bacteria were not spun onto the human Mϕ monolayers). The Pil^- strain associated with the human monolayers well (75% of control [Fig. 2A]), suggesting that nonopsonic phagocytosis of *P. aeruginosa* PAK by human macrophages is not fully dependent on the presence of this ligand. Overall, these data suggest that similar receptor-ligand interactions occur between either human or murine macrophages and *P. aeruginosa*.

Human neutrophils were isolated and purified by density gradient centrifugation as described previously (7). Neutrophils were diluted to a final concentration of 4.4×10^5 cells per

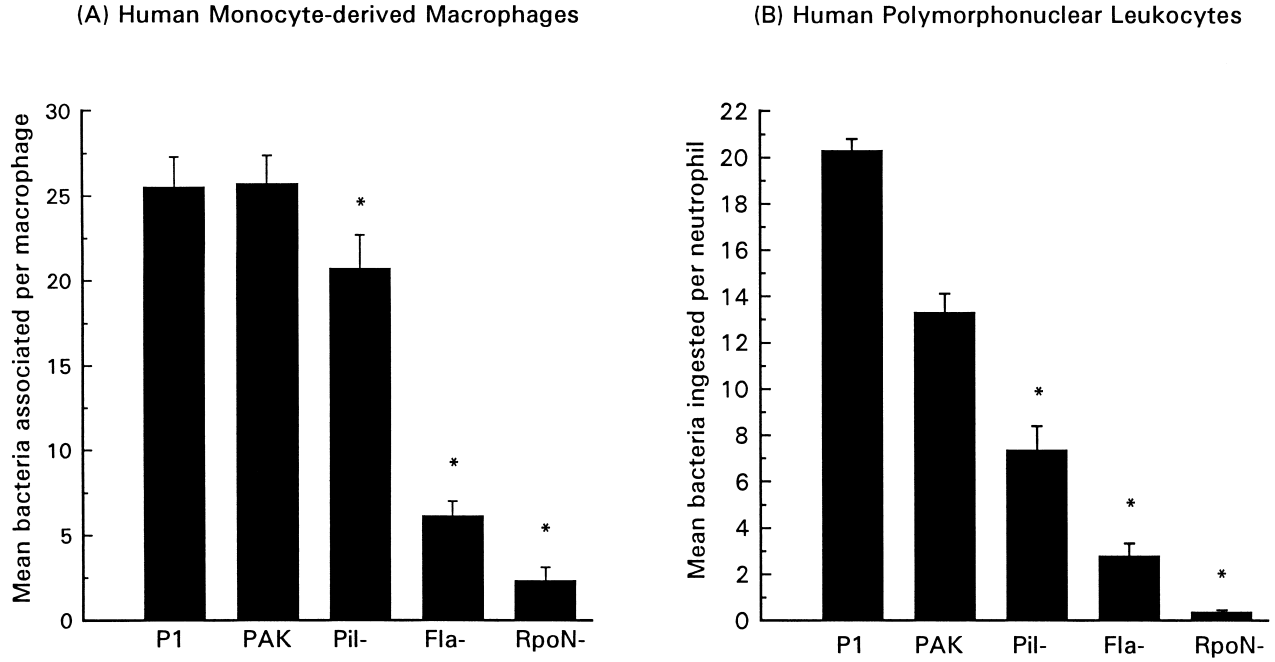

FIG. 2. Phagocytosis of *P. aeruginosa* P1 and PAK and its isogenic mutants by human Mϕ and PMNs. Error bars indicate the SEM ($n = 4$ experiments [A] and 6 experiments [B]), and the asterisk indicates significant difference compared with the value for the PAK control ($P \leq 0.05$).

ml in Hanks balanced salt solution (GIBCO-BRL, Gaithersburg, Md.) containing 0.1% gelatin (gHBSS), and 450-μl aliquots (2×10^5 cells) were dispensed into 5-ml sterile plastic tubes. After 30 min of incubation at 37°C with end-over-end tumbling, approximately 3×10^7 bacteria (an infection ratio of approximately 140 bacteria per neutrophil) were added and the incubation was continued for 1 h. The cells were then diluted with 2.5 ml of gHBSS, and the neutrophils were collected by gentle centrifugation at $200 \times g$ for 10 min at room temperature. After resuspension in 1 ml of gHBSS, 100 μl of the suspension was deposited onto a glass slide by cytocentrifugation (Cytospin 2; Shandon Southern Products Ltd., Astmoor, England). The slides were stained with 3% Giemsa, and phagocytosis was assessed by light microscopy as described above (ingested bacteria inside phagosomes were clearly visible after cytocentrifugation).

Nonopsonic interaction of the PAK mutants with human PMNs mimicked that of the murine macrophages. All isogenic mutants were ingested to a significantly lesser extent than the parental strain. Phagocytosis was dependent on the presence of both pili and flagella, and the RpoN mutant was almost completely resistant to uptake (Fig. 2B). The lack of ingestion by PMN of the PAK Fla$^-$ mutant was probably not due to reduced bacterium-phagocyte contact associated with the absence of bacterial motility; the PMN phagocytic assay was performed in a small volume of medium with end-over-end mixing of the contents, and these conditions are expected to promote contact between bacteria and phagocytes. Resistance to ingestion of the flagellin mutant in the neutrophil assay and in the forced-contact murine macrophage assay suggests a critical role for the flagellum in the nonopsonic binding and uptake of *P. aeruginosa* by phagocytes. Partial restoration of phagocytic susceptibility was achieved when the flagellin mutant was complemented with the wild-type gene, confirming the role of the flagellum in nonopsonic ingestion. Insertional inactivation of flagellin expression may have considerable polar effects since the gene is part of a regulatory hierarchy of genes required for synthesis of the flagellum and motility in *P. aeruginosa*. Therefore the moiety responsible for stimulation of nonopsonic phagocytosis may be a factor associated with synthesis of the flagellum structure which is not expressed if flagellin is not secreted.

The role of pili in the nonopsonic uptake of *P. aeruginosa* by macrophages has been demonstrated in several studies (3, 5, 13), although not one of these studies distinguished between the binding and subsequent ingestion events (as we have with the lysozyme washing procedure). Kelly et al. (3) used a pilus-deficient *pilA*::Tn*501* mutant of *P. aeruginosa* PAO1 to demonstrate that fibronectin-stimulated nonopsonic phagocytosis of *P. aeruginosa* by a macrophage-like murine cell line was a pilus-mediated event. However, the studies we report here suggest that nonopsonic phagocytosis is a very complex process requiring bacterial pili, nonpilus adhesins, and flagella, the latter being necessary for internalization. Mork and Hancock (5), utilizing the fibronectin-stimulated phagocytic assay (3), demonstrated that a spontaneous flagellum-deficient mutant of *P. aeruginosa* PAO1 was able to bind to macrophages; they were unable to show that this binding led to subsequent uptake of the bacterium. Earlier studies from our laboratory, utilizing *P. aeruginosa* CF isolates, indicated that association of the bacterium with phagocytes in the absence of serum opsonins was correlated with the presence of pili and the ability of the organism to form a surface pellicle when grown in static broth (13); the present study also correlates susceptibility of *P. aeruginosa* to nonopsonic phagocytosis with pellicle formation, a phenotypic feature of motile isolates (Table 1). Our study indicates that the flagellum not only is required to mediate adequate contact for binding to macrophages but also has a role in stimulating the ingestion of *P. aeruginosa* by murine macrophages.

Flagellum-mediated motility has been shown to play a role in the adherence of *P. aeruginosa* to respiratory epithelia in vitro (8, 9); those studies did not, however, demonstrate a specific binding interaction between the flagellum and respiratory-cell surfaces. Similarly, our study suggests that bacterial motility is required for efficient binding of *P. aeruginosa* to phagocytes by pilus and nonpilus adhesins. However, forcing contact between bacteria and macrophages suggested that motility was necessary but not sufficient for ingestion to occur and that the flagellum is also required for internalization of *P. aeruginosa*. Preston and King (6) demonstrated flagellum-dependent binding and endocytosis of soil pseudomonads by soil amoebas. Recently, Yao et al. (17), using insertional mutants, demonstrated that adherence and invasion of eukaryotic cells by *Campylobacter jejuni* was dependent on the presence of the flagellum. A role for the flagellum in specific binding and penetration of cell surfaces is therefore not unprecedented, and the flagellum-dependent internalization of *P. aeruginosa* warrants further investigation.

In conclusion, we have demonstrated that RpoN-dependent surface components of *P. aeruginosa* play a major role in mediating the nonopsonic phagocytosis of the organism by both macrophages and neutrophils. Although pilus and nonpilus adhesins and bacterial motility were required for binding of *P. aeruginosa* to phagocytic cells, the bacterial flagellum was required to trigger phagocytic internalization. We believe that the persistence of *P. aeruginosa* during chronic CF lung infection involves several phenotypic alterations that may enable the organism to evade phagocytic clearance; these include upregulation of the expression of mucoid exopolysaccharide and the loss of expression of RpoN-dependent surface ligands (pili and flagella).

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