INFECTION AND IMMUNITY, Aug. 1993, p. 3287–3293
0019-9567/93/083287-07$02.00/0

Vol. 61, No. 8

# Mechanisms of Nonopsonic Phagocytosis of *Pseudomonas aeruginosa*

THEYNE MORK AND ROBERT E. W. HANCOCK*

*Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3*

Received 14 December 1992/Accepted 12 May 1993

**The interaction of the macrophage cell line $P388_{D1}$ with *Pseudomonas aeruginosa* in the absence of stimulators or opsonins led to substantial association of bacteria, as judged by visual counting and FACScan assays. This association was observable within 5 min of addition of bacteria, could not be disturbed by exhaustive washing, and occurred with pilus- or flagellum-deficient mutants but not with *rpoN* mutants, which have been proposed to lack a secondary adhesin. In contrast, specific antibody was capable of causing similar enhancement of bacterial uptake regardless of the *rpoN* phenotype. Fibronectin stimulated uptake of bacteria with the pilus as an adhesin, and stimulation was observable within 5 min. Both fibronectin-enhanced and antibody-opsonized uptake were susceptible to inhibition by pertussis toxin but not by cholera toxin. The influence of fibronectin on $P388_{D1}$ cells was distinguishable from that of lipopolysaccharide, which caused substantial morphological changes in cells. Although lipopolysaccharide stimulated bacterial uptake, it actually suppressed fibronectin-mediated enhancement of uptake at high concentrations.**

Macrophages are an integral component of defenses against bacterial colonization and infection (7, 23, 24). They are involved in phagocytosis but also have an important role in amplification of host immune responses, mediation of inflammatory responses, and processing and presentation of antigens. *Pseudomonas aeruginosa* is susceptible to phagocytic killing by macrophages in both the presence and absence of opsonins, such as specific antibody (7, 15, 26). It was previously demonstrated that exposure of macrophages to fibronectin resulted in subsequent enhancement of nonopsonic phagocytosis of *P. aeruginosa* (14, 15, 29). This occurred regardless of the fibronectin source and with the $P388_{D1}$ macrophage cell line, with nonelicited mouse peritoneal macrophages, and with human monocyte-derived macrophages (15). The tetrapeptide RGDS from the eukaryotic cell-binding domain of fibronectin mimicked the action of intact fibronectin, and stimulation resulted from the action of fibronectin on macrophages and not from the direct involvement of fibronectin as an opsonin (16).

The bacterial ligand involved in fibronectin-stimulated nonopsonic phagocytosis was the bacterial pilus, and it was proposed that fibronectin upregulated the exposure of pilus receptors in macrophages and macrophage cell lines (14). However, our previous studies suggested that there was a substantial background association of *P. aeruginosa* with macrophages that was not pilus mediated or stimulated by fibronectin (14, 15). In this study, we have investigated the mechanisms of nonopsonic phagocytosis, with particular reference to fibronectin stimulation.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *P. aeruginosa* strains used were the wild-type strain H103 (15) and its nonmotile, rough, piliated derivative H237, kindly provided by A. Kropinski (Department of Microbiology, Queen's University, Ontario, Canada) and the PAK parental wild-type strain and its *rpoN* mutant derivative NIG, which carries a gentamicin resistance cassette in the *rpoN* gene (4), kindly provided by S. Lory (Department of Microbiology, University of Washington, Seattle). The phenotypes of strain H237 were confirmed on motility test medium and by pilus-specific phage adsorption.

Strains were maintained on Trypticase soy broth agar (Becton Dickinson & Co., Cockeysville, Md.) between assays. Twenty-four hours prior to the FACScan and phagocytosis assays, the strains were spread-plated onto BM2-glucose agar (8) with 0.02 mM $(NH_4)_2SO_4$ (low-nitrogen BM2) to increase piliation of the bacteria (13) and incubated at 37°C. Twenty-four-hour cultures were resuspended to a concentration of $10^9$ bacteria per ml in phosphate-buffered saline (PBS, pH 7.2) (14) for use in all phagocytosis assays.

To prepare bacteria for the FACScan assays, 1 mg of fluorescein isothiocyanate (FITC; Sigma, St. Louis, Mo.) in 3 ml of PBS was spread onto low-nitrogen BM2-glucose agar plates. Bacteria ($10^9$ cells per plate) were then plated onto this medium, and the plates were wrapped in tin foil and incubated for 24 h at 37°C. All FITC manipulations were performed in a darkroom under safelight conditions. After incubation, the FITC-labeled bacteria were suspended to a concentration of $10^{10}$ bacteria per ml in 1% bovine serum albumin (Boehringer, Mannheim, Germany) in PBS, pH 7.2, and the free FITC was separated from that bound to bacteria by sieving 1 ml of this suspension through a Sepharose 2B column (Pharmacia, Uppsala, Sweden). The fractions were collected in a darkroom under safelight conditions, and the fractions containing FITC-labeled bacteria were determined by their relative fluorescence with a Perkin-Elmer 650-10S fluorescence spectrophotometer. Fluorescence microscopy indicated that all cells were labeled uniformly and did not form aggregates. The labeled bacteria were resuspended to a concentration of $10^9$ bacteria per ml for use in the FACScan assay.

Bacteria (at a concentration of $10^9$/ml) were opsonized prior to use in the phagocytosis and FACScan assays by incubation with either antilipopolysaccharide (anti-LPS) monoclonal antibody MA1-8 (immunoglobulin G1 [IgG1] isotype) (9) from ascites for strain H103 or anti-PAK LPS

* Corresponding author.

(i.e., anti-serotype 6) serum (Sigma), at dilutions ($10^{-2}$ to $10^{-3}$) determined to be below the bacterial agglutination concentration. The antibody and bacteria suspensions were incubated with agitation for 30 min at 37°C prior to addition to $P388_{D1}$ cells in the assays.

**Phagocytosis assays.** A mouse macrophage cell line, $P388_{D1}$ (15, 17), was used in all experiments. The cells were cultured in Nunc flat-bottomed tissue culture flasks (Gibco, Burlington, Ontario, Canada) in RPMI 1640 medium plus glutamine supplemented with 10% fetal calf serum (GIBCO), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Terochem Laboratories, Vancouver, Canada), 44 mM sodium bicarbonate (Fisher Scientific, Vancouver, Canada), 0.04% (vol/vol) beta-mercaptoethanol (GIBCO), and 40 μg of gentamicin (Sigma; pH 7.2) per ml. The cells were incubated at 37°C in 7% $CO_2$.

Cells were gently scraped off the flask bottoms and resuspended at a concentration of $5 \times 10^5$ cells per ml in fresh RPMI 1640 medium prior to each assay. Trypan blue dye exclusion and a Petroff-Hauser chamber were used to count viable cells in the suspensions. Aliquots (2 ml) were plated into Nuclon 35-mm tissue culture dishes (GIBCO) and incubated overnight to give a confluent monolayer of $10^6$ cells. For early priming and time course experiments, 25-mm sterilized glass coverslips (VWR Scientific Inc., San Francisco, Calif.) were placed in the dish bottoms before the cell suspension was added. The overnight cultures were washed three times with unsupplemented RPMI 1640 medium, pH 7.2, to remove nonadherent cells before use in the assays. After removal of the nonadherent cells from the $P388_{D1}$ monolayers, 1 ml of unsupplemented RPMI 1640 was added to each tissue culture dish. Fifteen minutes prior to addition of bacteria, 100 μg of purified bovine plasma fibronectin glycoprotein, the cell-binding tetrapeptide RGDS (Sigma), or 100 μl of the pyrogen-free water used to resuspend the fibronectin or RGDS (Sigma) was added to the $P388_{D1}$ cell monolayers to assess enhancement of nonopsonic phagocytosis by the glycoprotein and its cell-binding region (16, 29).

Bacteria were added to the $P388_{D1}$ cell monolayers at a ratio of 20 bacteria per $P388_{D1}$ cell. For the early priming assays, the plates were swirled to distribute the bacteria, and the coverslips were removed 5 min postaddition. The coverslips were washed three times in unsupplemented RPMI 1640 medium, dried, fixed, and stained with Diff-Quik (Scientific Products, Baxter-Canlab, Mississauga, Ontario, Canada) for visual counting (30). In the other assays, the $P388_{D1}$ cells were allowed to incubate with the bacteria for 15 or 90 min. All incubations were performed at 37°C in 7% $CO_2$. The monolayers were washed three times with unsupplemented RPMI 1640, and the cells were lifted off the dish bottoms with a rubber policeman and resuspended in 1 ml of fresh RPMI 1640 by pipetting. Aliquots (100 μl) were spun at 450 rpm for 5 min onto glass microscope slides with a Cytospin 2 (Shandon Southern Instruments Inc., Sewickley, Pa.). The slides were Diff-Quik stained for visual counting with an Olympus BH-2 microscope (1,000×, oil immersion). For all visual counting assays, the bacteria bound to or within 90 phagocytes were counted for each slide. Statistical analysis of the data acquired was performed by Student's *t* test.

In experiments to test the influence of pertussis toxin (Sigma Chemical Co.) or cholera toxin (Calbiochem, San Diego, Calif.) on phagocytosis, the toxins were added according to previously published protocols (2, 3, 12). Purified (8) LPS, when used, was added to $P388_{D1}$ cells 30 min prior to addition of bacteria.

Lyophilized fibronectin reconstituted with endotoxin-free water was shown to contain less than 1 pg of endotoxin per 100 μg of sample by the *Limulus* amoebocyte lysate (LAL) test. Therefore, any enhancement of uptake by the addition of fibronectin was due to the influence of this molecule and not to contamination by endotoxin. The RPMI medium showed a strong positive reaction (>50 μg/100 ml) in the LAL test; however, the buffer did not appear to stimulate the $P388_{D1}$ cells, as judged from their lack of the morphological changes described in Results and the observation that LPS added to a final concentration 10 ng/ml caused maximal stimulation of phagocytosis, whereas concentrations higher than 10 μg/ml caused inhibition. Thus, we conclude that the LAL-positive material in RPMI medium was not LPS. Control experiments with dansyl polymyxin as an LPS probe (19) failed to demonstrate any LPS in water that gave a similar positive LAL test.

**FACScan assay of phagocytic activity.** To provide a rapid phagocytosis assay with a greater population sampling capacity, a flow cytometry method was devised from published procedures (1). $P388_{D1}$ monolayers were prepared as previously described. For some assays, the cells were pretreated with 1 μg of pertussis toxin as above. Antibody-opsonized or nonopsonized FITC-labeled bacteria were added to the monolayers at a ratio of 20 bacteria per phagocyte. All manipulations were performed in a darkroom under safelight conditions. The tissue culture dishes were wrapped in tin foil and incubated at 37°C in 7% $CO_2$ for 90 min. After incubation, the monolayers were washed three times with unsupplemented RPMI 1640 to remove uningested bacteria and placed on ice to lift the cells from the plate bottoms. The cells were resuspended by pipetting, and 400-μl aliquots were placed in polystyrene round-bottomed tubes (12 by 75 mm; Becton Dickinson) in an ice bath. Two thousand cells from each aliquot were assayed for cell-associated FITC fluorescence as a measure of bacterial binding and uptake with a FACScan flow cytometer (Becton Dickinson). An internal standard of untreated $P388_{D1}$ cells was used to set the cytometry parameters for each assay.

## RESULTS

**Intrinsic binding of *P. aeruginosa* to macrophages.** Previous studies indicated a substantial background level of bacterial association with macrophages that was relatively independent of experimental conditions (14–16). This binding of 3 to 5 bacteria per macrophage occurred regardless of the source of the macrophages or the presence or degree of bacterial piliation. The binding did not appear to be due to improper washing techniques, since, in the studies reported here, washing or not washing the macrophage monolayers that had been incubated with bacteria prior to scraping off these monolayers and cytocentrifugation did not alter ($P > 0.5$ by Student's *t* test) the mean number of bacteria per cell or the standard deviation of the mean (4.7 ± 4.4 bacteria per macrophage).

To eliminate the possibility that bacteria were being trapped under the macrophages during cytocentrifugation, assays were performed after plating monolayers of $P388_{D1}$ cells onto glass coverslips (26), incubating these monolayers directly with bacteria, and washing them extensively prior to counting cell-associated bacteria. Although background numbers of bound bacteria were not reduced in this assay, a population of $P388_{D1}$ cells which were apparently nonphagocytic was revealed by visual inspection (Fig. 1). These cells were round, with little visible cytoplasm, which distinguished them from the rounded forms described by Stuart et

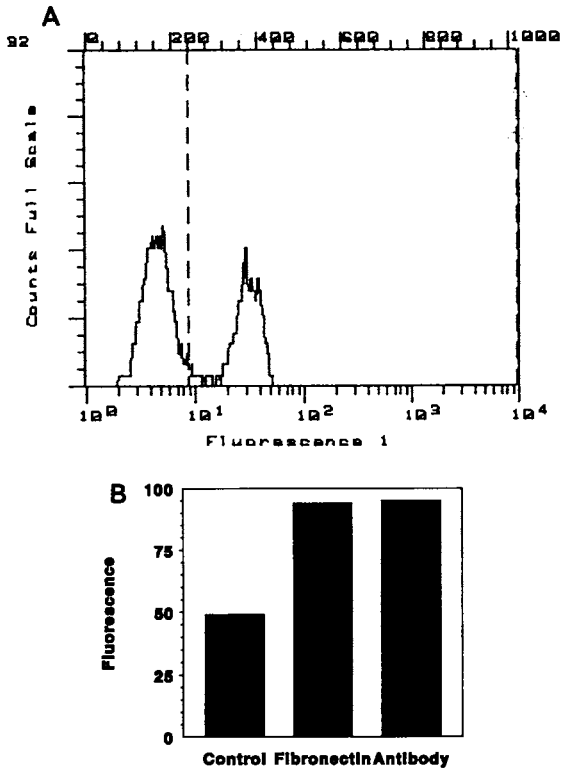


FIG. 1. Flow cytometry of FITC-labeled *P. aeruginosa* H103 association with $P388_{D1}$ cells. (A) Sample FACScan histogram of antibody-opsonized uptake. Two thousand phagocytes were assayed, and the dashed vertical line demonstrates the delineation between background fluorescence to the left and enhanced fluorescence due to bacterial association to the right, as determined statistically from controls without added bacteria. (B) Enhanced fluorescence due to bacterial association in the presence of water as a control, after 15 min of treatment with fibronectin or after incubation of bacteria with monoclonal antibody MA1-8. Statistical gating was used to eliminate the nonphagocytic population seen in panel A.

al. (27) as one of two morphological types seen in phagocytic cell cultures. Cytocentrifugation would flatten these cells, making them indistinguishable from spreading cells under the original protocol. Eliminating these cells from the visual counting process substantially lowered the standard deviation of the means for both the control and treated samples. This population of nonphagocytic cells was also observed in the FACScan assay of bacterial association with macrophages (Fig. 1A), with some variation between different phagocyte cultures in the number of nonphagocytic cells present. Such nonphagocytic cells have also been observed by other authors for $P388_{D1}$ cells (17) and alveolar macrophages (6), and FACScan analyses of IgG-mediated $P388_{D1}$ cell interaction with spleen cells have also indicated two populations of cells (25). Nevertheless, FACScan assays performed on suspended cells still revealed a level of nonopsonic association of bacteria that was approximately equal to that due to fibronectin stimulation or antibody opsonization (Fig. 1B). Control strain H103 cells grown under conditions that did not result in piliation (14, 15) prior to fluorescent labeling resulted in only a single peak of fluorescence that fell below the background value in the FACScan analysis.

**Evidence for involvement of a secondary adhesin.** It has been demonstrated that pili mediate binding of *P. aeruginosa* to macrophages and epithelial cells (4, 14, 26). However, pilus-deficient mutants still demonstrate significant binding (4), as also demonstrated for binding of pilus-deficient mutants to macrophages (14). From the data obtained with an *rpoN* regulatory mutant in which the gene had been interrupted by a gentamicin resistance gene cassette, Chi et al. (4) postulated the existence of a secondary adhesin for epithelial cells. Thus, *rpoN* mutants lacking pili, flagella, and this secondary adhesin demonstrated no binding to epithelial cells. We considered here the possibility that the background nonopsonic association discussed above was due to this secondary adhesin. The *rpoN* mutant strain NIG was significantly deficient in its ability to bind to or be ingested by $P388_{D1}$ cells ($P < 0.001$ by Student's $t$ test) compared with the wild-type PAK1 (Table 1). As expected (14), because of their inability to produce pili, fibronectin could not stimulate binding of these bacteria. However, opsonization by a polyclonal serum specific for strain PAK LPS resulted in uptake levels comparable to the antibody-enhanced levels (i.e., after background subtraction) of the wild-type PAK strain ($P > 0.5$ by Student's $t$ test).

TABLE 1. Effect of mutations influencing the expression of surface ligands on nonopsonic phagocytosis of *P. aeruginosa*

| Strain | Description | Mean no. of bacteria/phagocyte ± SD[a] with: | | |
|---|---|---|---|---|
| | | No addition | Fibronectin | Antibody[b] |
| H103 | Parental | 9.0 ± 5.8 | 17.1 ± 10.5[c] | 17.6 ± 8.5[c] |
| H237 | Flagellum deficient | 6.0 ± 6.3 | 13.9 ± 8.4[c] | ND[d] |
| PAK | Parental | 10.6 ± 6.7 | 20.7 ± 11.6[c] | 22.7 ± 12.3[c] |
| NIG | *rpoN* mutant[e] | 0.6 ± 0.9[f] | 1.5 ± 0.9[f] | 10.2 ± 5.7[c] |

[a] Data are from three separate experiments.
[b] Antibodies used were anti-LPS monoclonal antibody MA1-8 for strain H103 and polyclonal antibody specific for PAK LPS for strains PAK and NIG.
[c] Significantly ($P < 0.01$) different from the equivalent no-addition control value.
[d] ND, not determined.
[e] *rpoN* mutants lack flagella, pili, and a secondary adhesin.
[f] Significantly ($P < 0.001$) different from the value for the parent strain PAK.

The question arose whether the absence of flagella could account in part for the reduction in binding. Motility appeared not to be a factor in the kinetics of uptake at 90 min, since nonmotile mutants of strain H103 which expressed pili but not flagella were not significantly reduced in their ability to bind to $P388_{D1}$ cells ($P > 0.5$ by Student's $t$ test) (Table 1). Uptake of these mutants was subject to enhancement by fibronectin treatment of the $P388_{D1}$ cells. These data suggested that background uptake may result from attachment via the secondary *rpoN*-controlled adhesin.

**Early priming effects of fibronectin.** In an attempt to minimize the background and study fibronectin's influence on early binding kinetics, monolayers of $P388_{D1}$ cells on 25-mm coverslips were sampled at an early time point, 5 min after addition of bacteria. Cells were treated for 15 min with either pyrogen-free water or 100 μg of purified bovine plasma fibronectin reconstituted with the same pyrogen-free water. After 5 min of incubation with *P. aeruginosa* PAO strain H103, the coverslips were removed, washed, and stained for visual counting of cell-associated bacteria. These experiments demonstrated that binding of *P. aeruginosa* H103 by these phagocytic cells was an early phenomenon influenced by the presence of fibronectin (Table 2). Fibronectin increased not only the number of bacteria bound

TABLE 2. Influence of fibronectin treatment on association of *P. aeruginosa* H103 with $P338_{D1}$ cells[a]

| Treatment | No. of $P388_{D1}$ cells counted | Mean no. of bacteria/$P388_{D1}$ cell ± SD[b] | % of $P388_{D1}$ cells binding bacteria |
|---|---|---|---|
| PBS | 258 | 1.6 ± 1.6 | 72 |
| Fibronectin | 239 | 3.4 ± 2.1[c] | 94 |

[a] $P388_{D1}$ cells were treated with 100 μg of bovine plasma fibronectin in 100 μl of PBS or with 100 μl of PBS. The assay was performed 5 min after the bacteria were added to the cells.

[b] Means ± standard deviations for all cells observed in three independent experiments. Nonphagocytic cells with rounded shapes (see text) were deleted from consideration.

[c] Significantly higher than value for PBS control ($P < 0.001$ by Student's $t$ test).

by each phagocyte but also the overall percentage of $P388_{D1}$ cells binding bacteria at 5 min. Thus, fibronectin appeared to enhance phagocytosis of bacteria by "priming" the phagocytic cells for increased binding, possibly through upregulation of their pilus receptors. However, even at this earliest time point, the background nonopsonic association described above was observed. Similar data were also recorded for strain PAK.

**Comparison with LPS.** The bacterial component LPS has potent biological activity, and its effects on several immune functions and their underlying mechanisms, including macrophage activation, have been described (10, 20, 30). To test whether fibronectin manifested its stimulatory effects on nonopsonic phagocytosis via mechanisms independent of the LPS pathway, comparative studies of the stimulation of phagocytosis by fibronectin and LPS purified from the *P. aeruginosa* wild-type strain H103 were performed.

Addition of *Pseudomonas* LPS to phagocytes enhanced uptake of strain H103 (Fig. 2). Enhancement of uptake was influenced by the LPS concentration and did not exceed that by fibronectin ($P > 0.2$ by Student's $t$ test). Maximal enhancement of uptake occurred at all concentrations tested between 10 ng/ml and 10 μg/ml (including 100 ng/ml and 1 μg/ml), with greater concentrations (100 μg/ml) actually decreasing uptake ($P < 0.001$) (Fig. 2). The effects of LPS and fibronectin did not appear to be significantly additive ($P > 0.1$); indeed, it appeared that LPS suppressed fibronectin enhancement. Similar results were observed when RGDS was substituted for fibronectin. $P388_{D1}$ cells treated with LPS or LPS and fibronectin displayed morphologies unlike those of cells treated with fibronectin alone. The majority of LPS-treated phagocytes were highly vacuolated, with most of the cell-associated bacteria contained intracellularly within these vacuoles (Fig. 3). However, LPS had no effect on cell viability in the time span of these experiments, as judged from continued adherence and phagocytic ability. Large numbers of vacuolate cells were not present in fibronectin-treated cells, and bacterial binding was evident without this characteristic. Thus, it appeared that fibronectin was acting on the phagocytes via mechanisms separate from those of LPS.

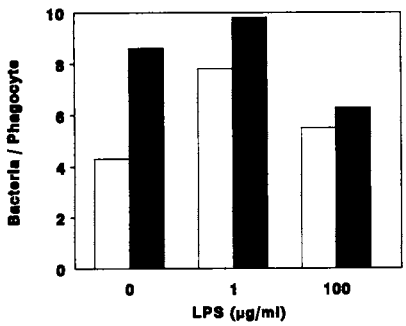


FIG. 2. Comparison of the effects of treatment with either purified *P. aeruginosa* H103 LPS for 30 min, bovine plasma fibronectin for 15 min, or both LPS and fibronectin for 30 min prior to addition of *P. aeruginosa* H103 on bacterial uptake by $P388_{D1}$ cells. Open bars, no fibronectin; shaded bars, 100 μg of fibronectin per ml. The level of LPS used is presented on the $x$ axis. The bacteria-to-phagocyte input ratio was 20:1, and the incubation time was 90 min. Data are representative of three separate experiments measuring bacterial association by direct counting.

**Toxin inhibition.** Many signal transduction pathways of macrophage maturation and activation have been shown to involve guanine nucleotide-binding proteins (G proteins). Many of these G proteins are substrates for cholera and/or pertussis toxin. Administration of these toxins to phagocytic cells either inhibits or promotes cellular responses for systems which utilize their substrate G proteins as the signal transducers (11). For example, Brown et al. (3) demonstrated the involvement of G proteins in opsonic phagocytosis by monocytes.

To probe for G protein function in fibronectin-stimulated nonopsonic phagocytosis, $P388_{D1}$ monolayers were incubated for 2 h with either cholera toxin or pertussis toxin prior to addition of *P. aeruginosa*. Cholera toxin at 5 μg/ml did not significantly alter the number of bacteria taken up by $P388_{D1}$ cells by either the opsonic, background nonopsonic, or fibronectin-stimulated nonopsonic mechanisms (Table 3).

Addition of 1 to 10 μg of pertussis toxin per ml significantly reduced the levels of both opsonic and fibronectin-stimulated nonopsonic uptake of bacteria for the phagocytes. At 1 μg of pertussis toxin per ml, the background nonopsonic uptake was also somewhat reduced, but the greatest reductions in uptake were seen for the fibronectin-treated and antibody-opsonized $P388_{D1}$ cells ($P < 0.001$ by Student's $t$ test) (Table 3). There was no apparent decrease in viability after toxin treatment at these concentrations, as judged from the continued adherence and normal morphology of the $P388_{D1}$ cells, in contrast to the results obtained in control experiments with cytocidal concentrations (100 μg/ml) of cytochalasin b, which caused morphological changes and cell liftoff. The visual assay results were confirmed by FACScan analysis, with substantial reductions in cell-associated fluorescence seen for both the fibronectin-treated and antibody-opsonized conditions.

## DISCUSSION

In this study, we explored the mechanisms involved in non-opsonic phagocytosis of *P. aeruginosa* with the mouse macrophage cell line $P388_{D1}$ as a model. Earlier studies (15) showed that this cell line provides an adequate model for unelicited mouse peritoneal macrophages and human monocyte-derived macrophages. It was previously demonstrated that fibronectin can stimulate macrophages to take up *P. aeruginosa* by a nonopsonic mechanism involving the bacterial pilus (14) and presumably the as yet unidentified macrophage pilus receptor. This fibronectin-stimulated, nonopsonic uptake likely equates to the nonopsonic uptake of *P. aeruginosa* observed by Speert et al. (26) with human monocyte-derived macrophages, since this also appears to be pilus-mediated uptake.

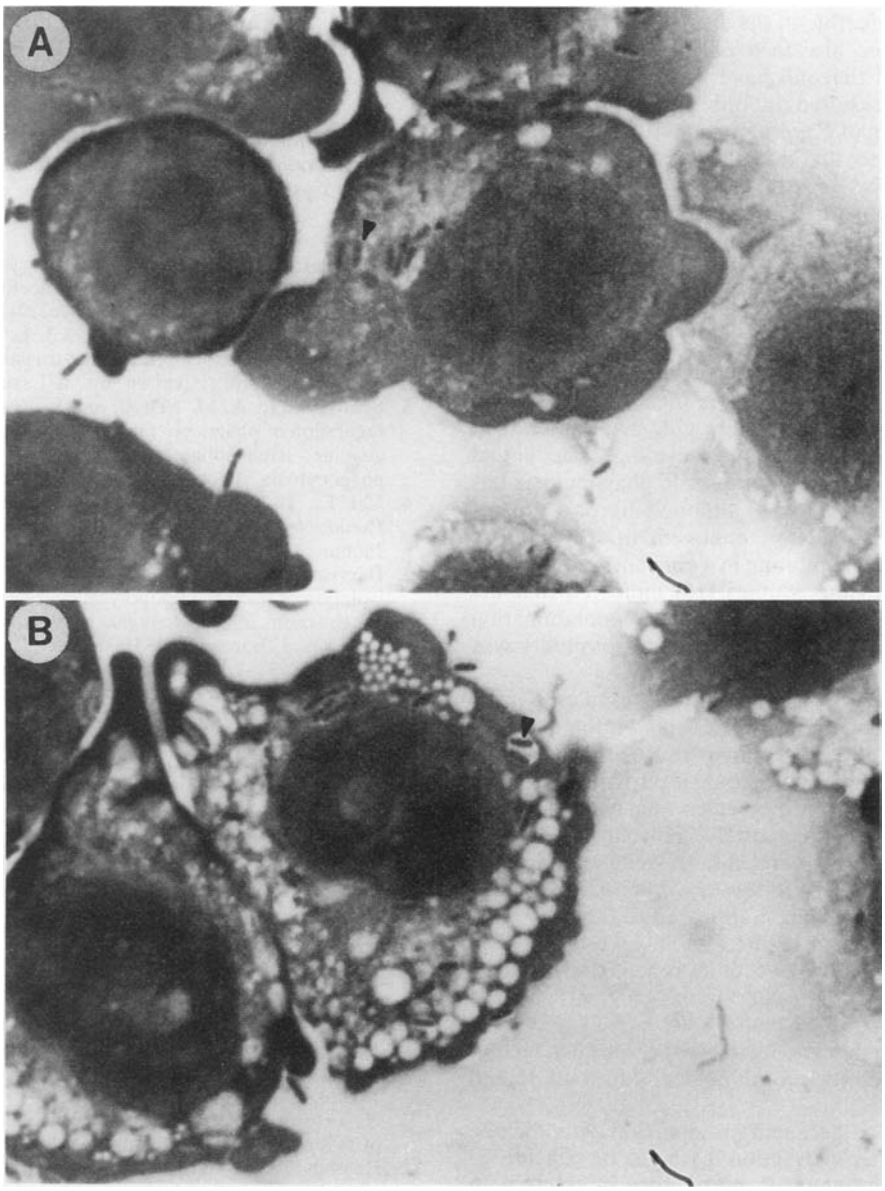


FIG. 3. Influence of LPS on $P388_{D1}$ cell morphology. (A) Control $P388_{D1}$ cell. (B) LPS (10 ng/ml)-treated $P388_{D1}$ cells. Arrowheads point to internalized *P. aeruginosa* bacteria.

TABLE 3. Effect of toxin treatment of $P388_{D1}$ cells on their ability to phagocytose *P. aeruginosa* H103

| Treatment (μg/ml) | Mean no. of bacteria/phagocyte ± SD[a] with: | | |
|---|---|---|---|
| | No addition | Fibronectin | Antibody |
| None (control) | 11.6 ± 6.8 | 19.7 ± 10.7[b] | 17.6 ± 8.1[b] |
| Cholera toxin (5) | 13.4 ± 8.8 | 20.0 ± 12.0[c] | 18.9 ± 9.1[c] |
| Pertussis toxin (1) | 7.2 ± 5.6[d] | 10.9 ± 7.5[d,e] | 9.7 ± 9.5[d,e] |

[a] Means ± standard deviations. Data are from three separate experiments.
[b] Significantly different ($P < 0.05$ by Student's $t$ test) from no-addition values.
[c] Significantly different ($P < 0.05$ by Student's $t$ test) from no-addition values.
[d] Significantly different ($P < 0.05$) from no-toxin values.
[e] Not significantly different ($P > 0.5$) from no-addition values.

In all previous experiments reported from our laboratory, a substantial background association of *P. aeruginosa* with macrophages was observed that was independent of fibronectin stimulation and unaffected by an insertion mutation leading to loss of pili (14, 15). This background association of bacteria with macrophages was not disturbed by washing, increased as a function of time, and was independent of growth under conditions that influenced the degree of bacterial piliation and hence fibronectin-stimulated nonopsonic phagocytosis (15). It was observed for both in vitro- and in vivo-grown bacteria (15). However, the results described here indicate that it was eliminated in an *rpoN* mutant which was reported to have lost a secondary nonpilus adhesin involved in binding of *P. aeruginosa* to epithelial cells (4). This was not due to an intrinsic inability of *rpoN* mutants to be phagocytosed, since antibody-opsonized phagocytosis was unaltered by this mutation. Although *rpoN*

mutants, which are defective in the sigma factor involved in low-nitrogen regulation, also lack pili and flagella, control experiments indicated that pili have no role (14), whereas flagellum deficiency resulted in only a 33% decrease in nonopsonic association of *P. aeruginosa* with cells (Table 1). Therefore, we propose that this background nonopsonic uptake is mediated by binding of *P. aeruginosa* via a nonpilus adhesin. Interestingly, this *rpoN*-regulated adhesin has been proposed to be involved in binding to respiratory mucins and thus may have a role in lung colonization by *P. aeruginosa* (22).

Fibronectin treatment was shown to influence the level of bacterial association with macrophages at the earliest sampling time (5 min). Therefore, we assume that fibronectin is acting to upregulate macrophage pilus receptors, as shown previously for complement receptors (28, 29), rather than influencing, for example, receptor recycling. The actual mechanism of stimulation and nonopsonic phagocytosis has received little attention to date, although fibronectin has been demonstrated to stimulate the movement of $K^+$ across the macrophage plasma membrane in a concentration-dependent manner (16). Previous studies (16) indicated that the effects of fibronectin were not explained by contamination with LPS, as confirmed here. However, this possibility was examined in more detail in the present study.

LPS has been shown by others to activate macrophages and to have more-global stimulatory effects on the mammalian immune system (10, 30). Comparison of the effects of fibronectin on $P388_{D1}$ cells with those of purified *P. aeruginosa* LPS showed that both molecules enhanced bacterial uptake when administered separately. Fibronectin (and its cell-binding region RGDS) were able to enhance bacterial association with $P388_{D1}$ cells to levels similar to those seen after LPS treatment; however, it appeared to function via a mechanism separate from that of LPS. This was evidenced by the antagonistic effects of LPS on fibronectin stimulation of phagocytes and by the gross morphological changes apparent in the LPS-treated cells. $P388_{D1}$ cells receiving LPS appeared to be highly vacuolated, whereas cells receiving fibronectin differed little morphologically from untreated cells.

The studies described here and previously have indicated four types of phagocytic interaction that must be considered in any circumstance in which *P. aeruginosa* interacts with macrophages. One, called here background nonopsonic association, occurs with $P388_{D1}$ cells grown in tissue culture, with nonelicited mouse peritoneal macrophages, and with human monocyte-derived macrophages (15). This association appears to involve an *rpoN*-controlled nonpilus bacterial adhesin and is somewhat sensitive to inhibition by pertussis toxin but not by cholera toxin. Such nonopsonic association occurs very soon after mixing of bacteria and macrophages. A second, LPS-stimulated nonopsonic association may be related to the first, although no specific studies were performed here. However, LPS-stimulated macrophages had striking morphological changes not observed under the other circumstances studied here. The third mechanism, fibronectin-stimulated nonopsonic uptake, involves pilus as the adhesin (14). It was sensitive to inhibition by pertussis toxin and to suppression by LPS treatment but was unaffected by cholera toxin. The fourth mechanism of phagocytic interaction, antibody-opsonized uptake, also showed inhibition by pertussis toxin in previous studies (3), as confirmed here. Thus, macrophages demonstrate a great deal of flexibility in dealing with *P. aeruginosa*. This may help to explain why infections by this bacterium are rarely observed in healthy individuals.

## ACKNOWLEDGMENTS

This research was supported by a grant to R.H. from the Medical Research Council of Canada and a studentship to T.M. from the Canadian Cystic Fibrosis Foundation.

## REFERENCES

1. **Bjerknes, R.** 1984. Flow cytometric assay for combined measurement of phagocytosis and intracellular killing of *Candida albicans*. J. Immunol. Methods **72**:229–241.
2. **Bokoch, G. M., L. A. Skleer, and J. E. Smolen.** 1987. Guanine nucleotide regulatory proteins as transducers of receptor-stimulated neutrophil activation. Int. J. Tissue Reactions **9**:285–293.
3. **Brown, E. J., A. M. Newell, and S. Gresham.** 1987. Molecular regulation of phagocyte function: evidence for involvement of a guanine triphosphate-binding protein in opsonin-mediated phagocytosis by monocytes. J. Immunol. **139**:3777–3782.
4. **Chi, E., T. Mehl, D. Nunn, and S. Lory.** 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infect. Immun. **59**:822–828.
5. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for the isolation of bacterial lipopolysaccharides from both smooth and rough strains of *Pseudomonas aeruginosa* and *Salmonella typhimurium*. J. Bacteriol. **155**:831–838.
6. **Davis, W. B., I. S. Barsoum, P. W. Ramwell, and H. Yeager.** 1980. Human alveolar macrophages: effects of endotoxin in vitro. Infect. Immun. **30**:753–758.
7. **Dunn, D. L., R. A. Barke, N. B. Knight, E. W. Humphrey, and R. L. Simmons.** 1985. Role of resident macrophages, peripheral neutrophils, and translymphatic absorption in bacterial clearance from the peritoneal cavity. Infect. Immun. **49**:257–264.
8. **Hancock, R. E. W., V. J. Raffle, and T. I. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19**:778–785.
9. **Hancock, R. E. W., A. A. Wieczorek, L. M. Mutharia, and K. Poole.** 1982. Monoclonal antibodies against *Pseudomonas aeruginosa* outer membrane antigens: isolation and characterization. Infect. Immun. **37**:166–171.
10. **Hara-Kuge, S., F. Amano, M. Nishijima, and Y. Akamatsu.** 1990. Isolation of a lipopolysaccharide (LPS)-resistant mutant, with defective LPS binding, of cultured macrophage-like cells. J. Biol. Chem. **265**:6606–6610.
11. **Harnett, M. M., and G. G. B. Klaus.** 1988. G protein regulation of receptor signalling. Immunol. Today **9**:315–319.
12. **Hensler, T., M. Raulf, F. Megret, J. E. Alouf, and W. Konig.** 1989. Modulation of leukotriene generation by pertussis toxin. Infect. Immun. **57**:3165–3171.
13. **Johnson, K., and S. Lory.** 1987. Characterization of *Pseudomonas aeruginosa* mutants with altered piliation. J. Bacteriol. **169**:5663–5667.
14. **Kelly, N. M., J. L. Kluftinger, B. L. Paslowske, W. Paranchych, and R. E. W. Hancock.** 1989. *Pseudomonas aeruginosa* pili as ligands for nonopsonic phagocytosis by fibronectin-stimulated macrophages. Infect. Immun. **57**:3841–3845.
15. **Kluftinger, J. L., N. M. Kelly, and R. E. W. Hancock.** 1989. Stimulation by fibronectin of macrophage-mediated phagocytosis of *Pseudomonas aeruginosa*. Infect. Immun. **57**:817–822.
16. **Kluftinger, J. L., N. M. Kelly, B. H. Jost, and R. E. W. Hancock.** 1989. Fibronectin as an enhancer of nonopsonic phagocytosis of *Pseudomonas aeruginosa* by macrophages. Infect. Immun. **57**: 2782–2785.
17. **Korer, H. S., B. S. Hanwerger, and J. R. Wunderlich.** 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. J. Immunol. **114**:894–897.
18. **Masanobu, O., M. S. Kang, and K. M. Yamada.** 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell **53**:649–657.
19. **Moore, R. A., W. C. Bates, and R. E. W. Hancock.** 1986.

Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl polymyxin. Antimicrob. Agents Chemother. **29:**496–500.
20. **Obi, C. L., and A. O. Coker.** 1989. The role of macrophages in experimental *Campylobacter* infections of mice in Lagos, Nigeria. Antibiot. Chemother. **41:**154–159.
21. **Proctor, R. A., J. A. Textor, J. M. Vann, and D. F. Mosher.** 1985. Role of fibronectin in human monocytes and macrophage bactericidal activity. Infect. Immun. **47:**629–637.
22. **Ramphal, R., L. Koo, K. S. Ishimoto, P. A. Totter, J. C. Lara, and S. Lory.** 1991. Adhesion of *Pseudomonas aeruginosa* pilin-deficient mutants to mucin. Infect. Immun. **59:**1307–1311.
23. **Rose, R. M.** 1989. Pulmonary macrophages in nosocomial pneumonia: defense function and dysfunction, and prospects for activation. Eur. J. Clin. Microbiol. Infect. Dis. **8:**25–28.
24. **Schaffner, T., H. V. Keller, M. W. Hess, and H. Cottier.** 1982. Macrophage functions in antimicrobial defense. Klin. Wochenschr. **60:**720–726.
25. **Segal, D. M., and D. A. Stephany.** 1984. The mechanism of intracellular aggregation. I. The kinetics of the $F_c\gamma$ receptor-mediated aggregation of $P388_{D1}$ cells with antibody-coated lymphocytes at 4°C. J. Immunol. **132:**1924–1930.
26. **Speert, D. P., F. Eftekhar, and M. L. Puterman.** 1984. Non-opsonic phagocytosis of strains of *Pseudomonas aeruginosa* from cystic fibrosis patients. Infect. Immun. **43:**1006–1011.
27. **Stuart, A. E., J. A. Habershaw, and A. E. Davidson.** 1970. Phagocytes *in vitro*. *In* D. M. Loc (ed.), Handbook of experimental immunology, 2nd ed. Blackwell Scientific Publications Ltd., Oxford.
28. **Wright, S. D., M. R. Licht, L. S. Criagmyle, and S. C. Silverstein.** 1984. Communication between receptors for different ligands on a single cell: ligation of fibronectin receptors induces a reversible alteration in the function of complement receptors on cultured human monocytes. J. Cell Biol. **99:**336–339.
29. **Wright, S. D., and B. C. Meyer.** 1985. Fibronectin receptor of human macrophages recognizes the sequence arg-gly-asp-ser. J. Exp. Med. **162:**762–767.
30. **Zimmerli, W., A.-M. Reber, and C. A. Dahinder.** 1989. The role of formyl peptide receptors, C5a receptors, and cytosolic-free calcium in neutrophil priming. J. Infect. Dis. **161:**242–249.