

Functional Characterization of Macrophage Receptors for In Vitro Phagocytosis of Unopsonized *Pseudomonas aeruginosa*

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Abstract

The phagocytic receptor for unopsonized *Pseudomonas aeruginosa* was characterized functionally using human monocyte-derived macrophages. Freshly isolated human peripheral blood monocytes were unable to ingest unopsonized *P. aeruginosa*; ingestion did not occur until the cells had been in culture for 2 d and it became maximal after 4 d. Macrophages plated on coverslips derivatized with anti-BSA IgG or with human gammaglobulin lost the capacity to phagocytose unopsonized *P. aeruginosa*, unopsonized zymosan, and EIgG but bound C3bi-coated erythrocytes normally. Each of the four human IgG subclasses and Fc fragments of anti-BSA IgG inhibited phagocytosis of both unopsonized *P. aeruginosa* and EIgG. Phagocytosis of *P. aeruginosa* and zymosan was markedly impaired and EIgG minimally inhibited if macrophages were plated on coverslips derivatized with mannan or when mannan was added to the phagocytosis buffer. Phagocytosis of *P. aeruginosa* and zymosan, and binding of EC3bi was dependent on the presence of divalent cations, but phagocytosis of EIgG was not. The macrophage phagocytic receptor for unopsonized *P. aeruginosa* was inactivated by proteolytic enzymes. Phagocytosis of *P. aeruginosa* was inhibited by D-mannose, L-fucose, and α methyl mannoside, but not by L-mannose, D-fucose, or D-glucose. The same sugars inhibited phagocytosis of unopsonized zymosan. We conclude that phagocytosis of unopsonized *P. aeruginosa* by human monocyte-derived macrophages is facilitated by mannose receptors.

Introduction

Although most pathogenic microorganisms require opsonization for efficient phagocytosis, certain bacteria, fungi, and parasites are recognized by macrophages or neutrophils in the absence of an exogenous source of complement or Ig (1, 2). We have observed previously that certain nonmucoid strains of *Pseudomonas aeruginosa* from patients with cystic fibrosis (CF)¹ are phagocytosed by human neutrophils (PMN), mono-

cyte-derived macrophages (M ϕ), and pulmonary alveolar macrophages in the absence of serum (3, 4). This process appears to be facilitated by bacterial piliation and hydrophobicity and is abrogated by the exopolysaccharide of mucoid strains (4, 5). Nonopsonic phagocytosis of *P. aeruginosa* by human PMN is inhibited by D-mannose and mannose-containing saccharides (3). Phagocytosis of zymosan by human macrophages is also mannose inhibitable and appears to be mediated predominantly by mannosyl, fucosyl receptors (MFR) (6).

The process of nonopsonic phagocytosis of *P. aeruginosa* may be of critical importance in the natural defense of the lung early in the course of infection. Before the evolution of an inflammatory response, there may be insufficient quantities of opsonins to permit phagocytosis by Fc or complement receptors, and nonopsonic receptor-mediated phagocytosis may play a key role in pulmonary antisepsis. In CF patients, *P. aeruginosa* colonizes and proliferates in the respiratory tract despite the presence of anti-pseudomonal Ig. In fact anti-pseudomonal IgG from some patients with CF inhibits phagocytosis of *P. aeruginosa* by macrophages (7, 8). Serum from some patients with CF may fail to promote phagocytosis and the killing of *P. aeruginosa* because of the epitope-specificity of the Igs (9). The purpose of this study was to characterize macrophage phagocytic receptors for unopsonized *P. aeruginosa* in order to better understand the process of normal pulmonary host defense against this organism and to gain insights into the pathophysiology of lung infections in patients with CF.

Methods

Reagents. Mouse MAbs against macrophage surface antigens were as follows: IB4 (IgG2a, anti- β chain of the C3bi receptor, LFA-1, p150,95 family) (10); OKM1 (IgG2a, anti- α chain C3bi receptor) (10); anti-Leu M5 (IgG2b, anti- α chain p150,95, Becton Dickinson & Co., Mountain View, CA) (11); anti-CR1 (IgG1, anti-C3b receptor, Becton Dickinson); Leu-15 (IgG2a, anti-C3bi receptor, Becton Dickinson); and 3G8 (IgG1, anti-Fc receptor of neutrophils and monocytes (12), kindly provided by Dr. Jay Unkeless, Mount Sinai Medical School, NY).

Rabbit anti-BSA was purchased from Cappel Biomedical, Inc. (Malvern, PA). Human Ig for intravenous use, pH 4.0 (Sandoglobulin) from Sandoz Canada Inc., contained > 90% monomeric IgG and all four human IgG subclasses. Human IgG subclasses were kindly provided by Dr. F. Skvaril, Institute for Cancer Research, Berne, Switzerland.

Mannan from *Saccharomyces cerevisiae*, D-mannose, L-mannose, α methyl D-mannopyranoside (α MM), D-fucose, L-fucose, and D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Fc fragments. Rabbit anti-BSA (6 mg/ml in 0.02 M PBS, pH 7.3) was incubated with 60 μ g/ml papain (Sigma) in 0.01 M cysteine and 0.002 M EDTA (Sigma) for 4 h at 37°C. After overnight dialysis in 0.005 M Tris-HCl, pH 8.0, the digestate was separated by ion-exchange using HPLC (Waters Associates, Milford, MA). Fc fragments formed crystals and were identified by molecular weight on

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1. Abbreviations used in this paper: α MM, α methyl D-mannopyranoside; CF, cystic fibrosis; EC3bi, erythrocytes opsonized with C3bi; EIgG, erythrocytes opsonized with IgG; Eth, sheep erythrocytes; GVB⁺, veronal buffer with 0.1% gelatin; MFR, mannosyl, fucosyl receptors; M ϕ , monocyte-derived macrophages.

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12.5% SDS-PAGE and by countercurrent immunoelectrophoresis using goat anti-rabbit IgG (Sigma). Fab and Fc fragments were readily distinguished by their characteristic migrations on immunoelectrophoresis.

Particles for phagocytic assays. *P. aeruginosa* strain P-1 NM is a spontaneous nonmucoid laboratory revertant of a mucoid strain from the sputum of a patient with CF (3). It is a LPS-rough, serum-sensitive, pilated strain. Bacteria were maintained frozen and grown fresh overnight on Mueller-Hinton agar for each experiment. Zymosan particles (Sigma) were prepared exactly as described (6).

Sheep erythrocytes (E^h) were purchased from Prepared Media Laboratories (Tualatin, OR) and were opsonized exactly as described previously (13). Briefly, erythrocytes opsonized with IgG (ElgG) were prepared by incubating E^h with a subagglutinating concentration of rabbit anti- E^h IgG (Cordis Laboratories, Inc., Miami, FL) at 37°C for 30 min and then at 0°C for 30 min. Erythrocytes opsonized with C3bi (EC3bi) were prepared by incubating E^h successively with rabbit anti- E^h IgM (Cordis), guinea pig C1, C4, C2 (Cordis), and human C3 (Cytotech, San Diego, CA), and finally with diisopropylfluorophosphate (Sigma) and human C3 inactivator (Cordis). ElgG were prepared fresh on the day of each experiment; the EC3bi were used within 2 wk of preparation.

Macrophage cultivation. Macrophages were derived from human peripheral blood monocytes by culture in suspension in teflon beakers as described previously (6). Briefly, mononuclear cells were separated from heparinized peripheral venous blood on a Ficoll-Hypaque gradient and cultured with 13% autologous fresh human serum at 37°C in 5% CO₂. The cells were washed free of serum on the day of each experiment and plated on glass coverslips. These cells were viable as determined by trypan blue dye exclusion and by their capacity to phagocytose opsonized and unopsonized particles.

Preparation of substrate-coated coverslips. Glass coverslips were derivatized with substrates by a modification of Michl's method (14). 12-mm-diam round glass coverslips were acid-washed and a 5-mm-diam circle was drawn in the center. The coverslips were placed, circle side down, in wells of a 24-well tissue culture plate (Falcon Labware, Becton Dickinson & Co., Oxnard, CA) and 0.5 ml of poly-L-lysine (mol wt > 70,000, 0.1 mg/ml) was added. After a 30-min incubation at 24°C, the coverslips were washed and incubated in 2.5% glutaraldehyde for 15 min at 24°C. The coverslips were then washed exhaustively, aspirated dry, and 10 μ l of the desired protein substrate was added to the center of the circle. After a further 60-min incubation at 24°C, the coverslips were washed and incubated in PBS with 1.5 g/100 ml glycine and 1 mg/ml BSA at 4°C overnight to block any unbound aldehyde sites. Immediately before use, the coverslips were washed in PBS, pH 7.4. Derivatization with polysaccharides was achieved using the same method except that 10 mg of 1-ethyl-3-(dimethylamino-propyl) carbodiimide was added to 50 mg of polysaccharide during the substrate binding step (15).

Assessment of phagocytosis. Phagocytosis of *P. aeruginosa* (3), zymosan (6), ElgG, and EC3bi (13) was assessed by visual inspection exactly as described. In most experiments, $M\phi$ were used between 4 and 7 d of in vitro cultivation, at which time they had all the characteristics of macrophages but had not begun to fuse with one another. In each experiment, 100 cells were examined. Phagocytosis of *P. aeruginosa* was expressed as the percentage of $M\phi$ with < 6, 6–19, or > 19 bacteria ingested. Phagocytosis of zymosan and ElgG was expressed as the average number of particles ingested by each $M\phi$. Results with EC3bi represented the average number of particles attached to each $M\phi$. All experiments were repeated at least three times on separate days with $M\phi$ from different donors. Results are expressed from individual experiments but are representative of data from the other days. Since the phagocytic capacity of $M\phi$ from different donors, or from the same donor on different occasions was so varied, pooling of data was not attempted.

Enzyme susceptibility of $M\phi$ receptors. Macrophages were plated on glass coverslips in the usual fashion, washed with PBS, and placed in separate wells of a 24-well tissue culture plate (Falcon). The wells

contained 1 ml of RPMI with 10 mM Hepes (Sigma) and one of the following enzymes (all purchased from Sigma): 100 and 500 μ g/ml trypsin, 100 μ g/ml pepsin, 10 and 100 μ g/ml protease type IX from *Bacillus polymyxa*, 10 and 100 μ g/ml papain, 10 and 100 μ g/ml neuraminidase, 100 μ g/ml α mannosidase, and 10 μ g/ml α galactosidase. After a 30-min incubation at 37°C in 5% CO₂, the coverslips were washed thoroughly with PBS and particles for phagocytosis were added to the wells.

Divalent cation-dependence for phagocytosis of different particles. Divalent cation-dependence was assessed either by adding Ca²⁺ and/or Mg²⁺ to cation-free media or by chelating the cations in the medium. GVB⁺ buffer (veronal buffer with 0.1% gelatin) was made with 2 g/liter BSA and 2.4 g/liter Hepes was added. In some experiments, 0.5 mM CaCl₂ and/or MgCl₂ was added to this buffer. $M\phi$ were washed, suspended in RPMI medium with 0.1% BSA and 10 mM Hepes, and plated on coverslips. After the $M\phi$ were plated, the coverslips were washed extensively in PBS without divalent cations and added to wells that contained GVB⁺ with or without the divalent cations. Particles were added and phagocytosis was assessed in the usual manner. For chelation of divalent cations, 2 mM EDTA or EGTA was added to RPMI medium with BSA and Hepes. Phagocytosis assays were performed in these solutions with $M\phi$ that had been plated on coverslips in RPMI medium with BSA and Hepes.

Electron microscopy. Macrophages were prepared as described above and plated in wells of a 24-well tissue culture plate (Falcon). Incubation with *P. aeruginosa* was conducted in the same way as it was for assessment of phagocytosis. After a 60-min incubation, the wells were washed and the contents were fixed with glutaraldehyde, osmium tetroxide, and graded concentrations of ethanol. The cells were then embedded in epon, sectioned, and viewed with a Jeol 1200 electron microscope.

Results

P. aeruginosa strain P-1NM was phagocytosed well by human monocyte-derived macrophages (4–6 d in culture) in the absence of exogenous serum opsonins. In contrast, freshly isolated human peripheral blood monocytes were unable to phagocytose *P. aeruginosa*; ingestion did not occur until the cells had been in culture for 2 d and maximal ingestion occurred after 4 d (Fig. 1). Electron microscopy performed on $M\phi$ which had been incubated in the presence of *P. aeruginosa* for 1 h at 37°C, revealed many bacteria within phagocytic vacuoles (Fig. 2A). Bacteria were also seen surrounded by pseudopods, with the $M\phi$ membrane in close contact with the engulfed microorganisms (Fig. 2B).

When macrophages spread on antibody- or ligand-coated surfaces, the receptors that bind to these antibodies or ligands are down-modulated to the adherent surface of the cell (14); the phagocytic function of that receptor is thereby depressed.

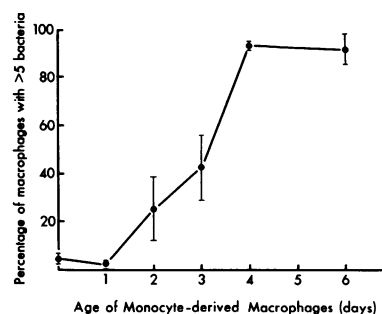


Figure 1. The relationship between maturation of human monocyte-derived macrophages and phagocytosis of unopsonized *Pseudomonas aeruginosa*. The phagocytic index (mean \pm SE of three experiments) represents the percentage of macrophages ingesting more than five bacteria.

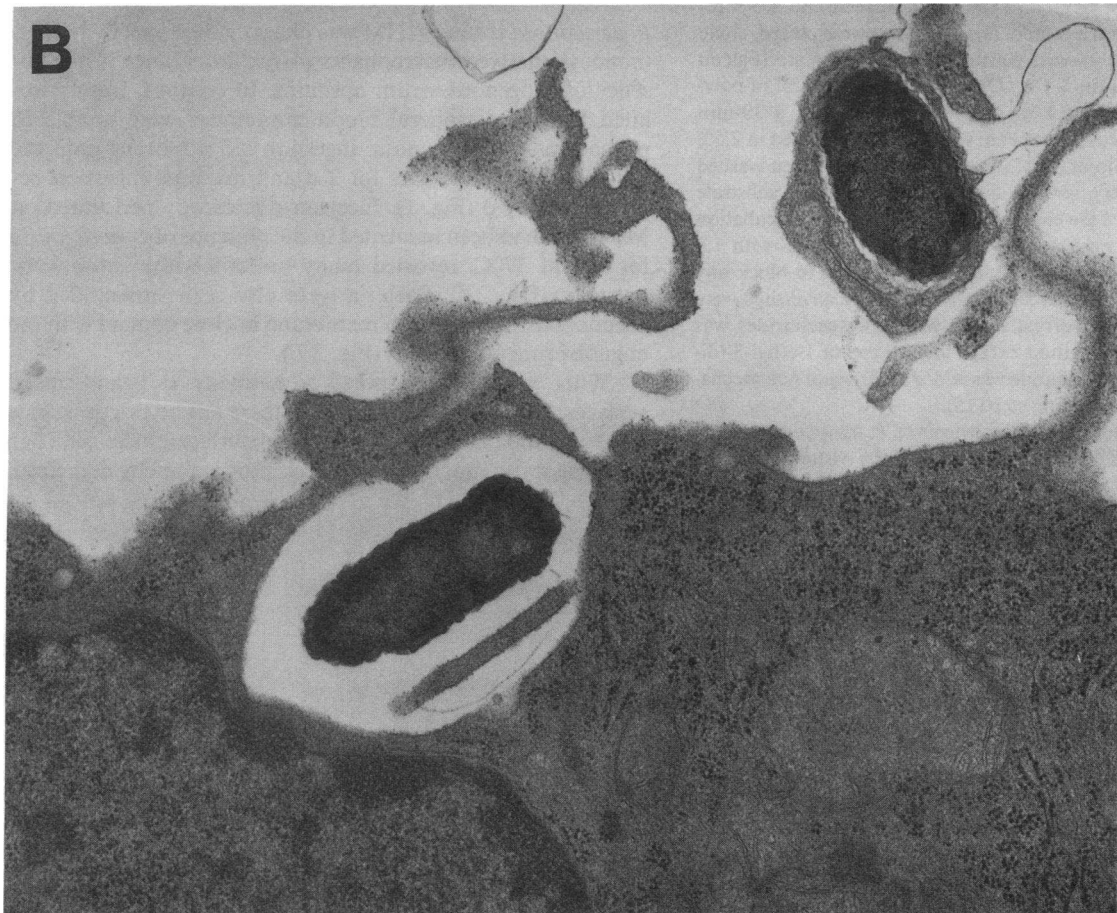
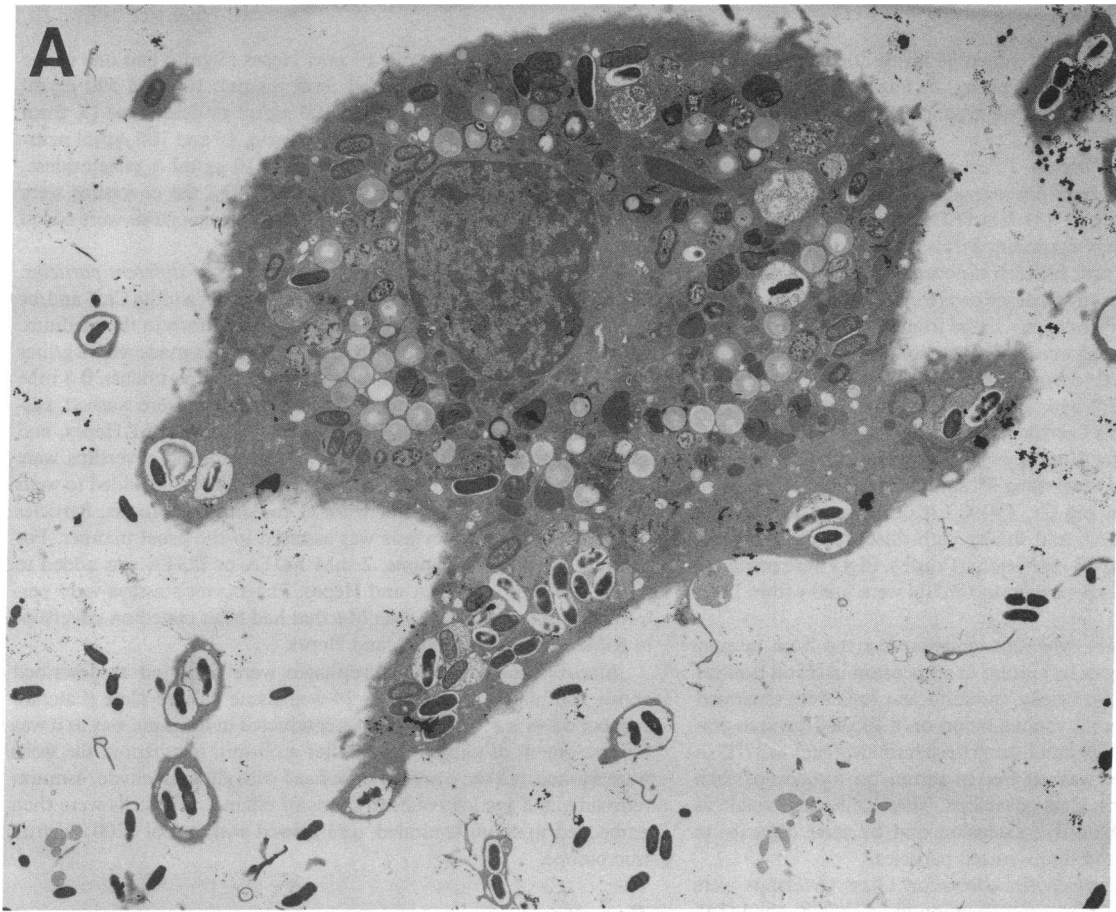


Table I. Phagocytosis of *P. aeruginosa* by Macrophages Plated on Antibody-coated Coverslips

Substrate	Phagocytosis*		
	<6	6–19	>19
BSA (control)	0	8	92
MAb IB4 (10 µg/ml)	46	40	14
MAb OKM1 (20 µg/ml)	4	24	72
MAb anti-Leu M5 (25 µg/ml)	16	16	68
MAb anti-CR1 (25 µg/ml)	12	15	73
MAb Leu 15 (20 µg/ml)	7	6	87
MAb 3G8 (20 µg/ml)	8	37	55

* Percentage of macrophages with the indicated number of bacteria ingested.

To determine if unopsonized *P. aeruginosa* is phagocytosed by one of the recognized macrophage membrane receptors, coverslips were derivatized with MAbs directed against the C3bi receptor (IB4, OKM1, and Leu 15), the C3b receptor (anti-CR1), a human PMN and monocyte Fc receptor (3G8), and the p150,95 antigen (anti-Leu M5). Macrophages were plated on the derivatized coverslips and then phagocytosis of *P. aeruginosa* was assessed. For the control, macrophages were plated on a coverslip derivatized with BSA. This experiment was performed on 18 separate occasions with macrophages (after 4–7 d of in vitro culture) from different donors. Each of the MAbs inhibited phagocytosis. MAb IB4 (directed against the beta chain of the C3bi receptor, LFA-1, and p150,95 family of glycoproteins) consistently exerted the greatest inhibitory effect. Results of one such experiment are shown in Table I.

These MAbs were bound to the coverslips, presumably in random orientations, with either the Fab or Fc portions available to ligate macrophage receptors. Since all of the antibodies inhibited the phagocytosis of unopsonized *P. aeruginosa* to varying degrees, it was possible that they did so via their Fc domains. Macrophages were therefore plated on coverslips derivatized with rabbit anti-BSA or human gammaglobulin. Neither of these antibody preparations is directed against macrophage membrane antigens; they would therefore be expected to specifically ligate only Fc receptors. Phagocytosis of *P. aeruginosa* and ElgG was inhibited when Mφ were plated on either antibody substrate, but binding of EC3bi was unaffected (Table II). These data suggest that the Fc domain of IgG is recognized by receptors that bind *P. aeruginosa*, or that the receptors that mediate *Pseudomonas* ingestion co-modulate with the Fc receptors.

To determine which of the human IgG subclasses was most capable of down-modulating receptors that recognize unopsonized *P. aeruginosa*, Mφ were plated on coverslips derivatized with a range of concentrations of each of the four subclasses. All four human subclasses were able to interfere with the phagocytosis of both unopsonized *P. aeruginosa* (Fig. 3 A) and ElgG (Fig. 3 B). Although the dose-response inhibition was not markedly different among the subclasses for either of

Table II. Phagocytosis or Binding of *P. aeruginosa* and Opsonized Erythrocytes by Macrophages Plated on Ig-coated Coverslips

Substrate	Phagocytosis			Binding
	<i>P. aeruginosa</i> *	Zymosan†	ElgG‡	EC3bi§
Control (BSA)	92	6.5	22.8	17.1
Anti-BSA (60 µg/ml)	53	3.0	2.0	20.1
Human gammaglobulin (30 mg/ml)	7	2.7	5.8	18.5

* Percentage of macrophages with > 19 bacteria ingested.

† Average number of particles ingested per macrophage.

‡ Average number of particles attached per macrophage.

the two particles, IgG2 was less effective than IgG1, 3, or 4 in down-modulating Fc receptor activity (Fig. 3 B).

To further confirm a requirement for the Fc domain of IgG to down-modulate macrophage receptors for *Pseudomonas*, Mφ were plated on coverslips derivatized with Fc fragments of anti-BSA IgG. Phagocytosis of both *P. aeruginosa* and ElgG was inhibited (Table III). Fc receptors are co-down-modulated with MFR when murine Mφ are plated on mannan-coated surfaces (16). Inhibition of both ElgG and *P. aeruginosa* phagocytosis could therefore indicate that they are both phagocytosed by the same receptor, that the receptors for the two particles are associated with one another and are thus co-down-modulated by a single ligand, or that Fc receptors and MFR both react with the same ligands.

Expression of MFR by Mφ is under maturational control (6); ingestion of zymosan does not occur until the cells have been in culture for at least 48 h. We observed that the capacity of Mφ to phagocytose *P. aeruginosa* exhibits a similar maturational dependence. To determine whether Fc receptors exhibited the same maturational characteristics in this phagocytic system, ingestion of ElgG by monocytes in culture on days 0–4 was assessed (Table IV). Freshly obtained human peripheral blood monocytes were able to ingest ElgG, as were cells after 1–4 d of in vitro culture, which confirmed our previous observation (12). These observations suggest that the MFR, not Fc receptors, recognize unopsonized *P. aeruginosa*.

To determine the role of MFR in the phagocytosis of unopsonized *P. aeruginosa*, Mφ were plated on coverslips that had been derivatized with mannan (a mannose-containing oligosaccharide with a potent capacity to ligate MFR). Phagocytosis of *P. aeruginosa* and zymosan was impaired markedly after Mφ were plated on mannan-coated coverslips but ElgG ingestion was inhibited only marginally. We observed a similar inhibition pattern of phagocytosis of those three particles when mannan was added to the phagocytosis buffer (Table V). Phagocytosis of *P. aeruginosa* and zymosan was reduced in proportion to the concentration of mannan in the medium. In contrast, uptake of ElgG was reduced by about the same amount (30–40%) regardless of the concentration of mannan

Figure 2. Electron micrographs of 5-d-old monocyte-derived macrophages incubated in the presence of unopsonized *Pseudomonas aeruginosa* for 1 h. (A) Bacteria in phagocytic vacuoles both within pseudopodia and perinuclear cytoplasm. (B) The macrophage membrane tightly affixed to a bacterium in an early stage of phagocytosis.

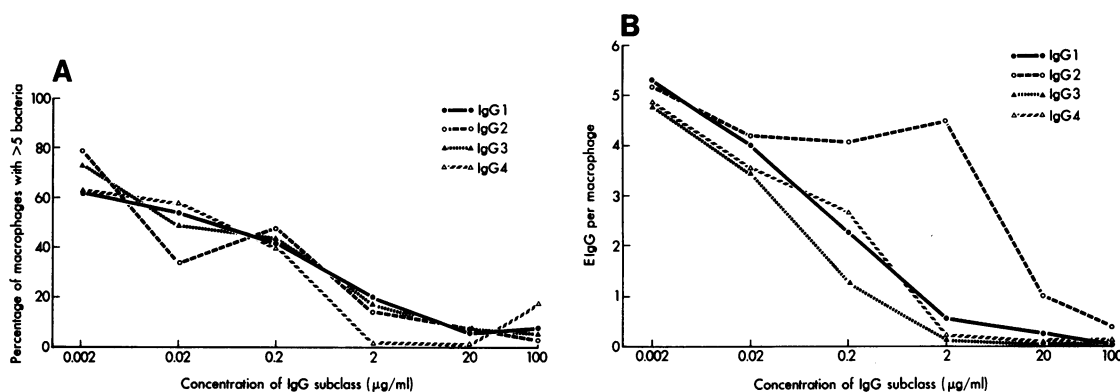


Figure 3. The effect of human IgG subclasses on phagocytosis of unopsonized *Pseudomonas aeruginosa* (A) and EIgG (B). Human monocyte-derived macrophages were plated on coverslips that were derivatized with different concentrations of each IgG subclass and phagocytosis was assessed (see Methods for details).

used. EIgG are phagocytosed by macrophage Fc receptors (1, 2) and zymosan by macrophage MFR (6) with the cooperation of CR3 (17). It appears, therefore, that IgG caused down-modulation of Fc receptors and MFR, and that mannan caused only modest impairment of Fc receptor function but profoundly inhibited MFR function. Further experiments were necessary to determine which of these two receptors might mediate *P. aeruginosa* uptake since both mannan and IgG inhibited its phagocytosis.

Each macrophage phagocytic receptor has characteristic functional requirements for divalent cations. Receptors for EC3bi and mannose require Ca^{2+} and/or Mg^{2+} , but receptors for the Fc domain of IgG mediate binding and phagocytosis of particles in a divalent cation-free system (1, 15, 18). Macrophage divalent cation-dependence for phagocytosis of *P. aeruginosa* was assessed by modifying the Ca^{2+} and/or Mg^{2+} content of the medium (Table VI). Divalent cations were chelated by adding EDTA or EGTA to the RPMI medium. Phagocytosis of *P. aeruginosa*, EIgG, and zymosan, and binding of EC3bi occurred normally in RPMI medium that contained both Ca^{2+} and Mg^{2+} . Phagocytosis of *P. aeruginosa* and of zymosan and binding of EC3bi was inhibited by chelation of divalent cations. Only EIgG was ingested in GVB⁻ (which is devoid of divalent cations), confirming that Fc receptors remain functional in the absence of divalent cations. Phagocytosis (*P. aeruginosa* and zymosan) or attachment (EC3bi) were enhanced when Ca^{2+} and Mg^{2+} were added to GVB⁻. Phagocytosis and/or attachment of all four particles was better in RPMI medium

than in GVB⁻ with divalent cations added. These data confirmed that receptors for EC3bi and zymosan were divalent cation-dependent, but that Fc receptors were not. They also showed that the receptors that mediated *P. aeruginosa* uptake resemble the C3bi receptor and MFR in their cation dependence.

The macrophage receptors for phagocytosis of unopsonized *P. aeruginosa* were further characterized by assessing their sensitivity to inactivation by different enzymes (Table VII). Treatment of macrophages with the proteolytic enzymes, trypsin, protease, and papain, inhibited phagocytosis of unopsonized *P. aeruginosa*. Phagocytosis was unaffected when macrophages were pretreated with α galactosidase, α mannosidase, pepsin, or neuraminidase. The receptors for binding of EC3bi and for phagocytosis of zymosan were also trypsin sensitive, but Fc receptors were not.

Finally, we assessed the ability of soluble monosaccharides to inhibit uptake of *P. aeruginosa* (Table VIII). D-mannose, L-fucose, and α MM were inhibitory, whereas L-mannose, D-fucose, and D-glucose were not. The same pattern of inhibition was seen for zymosan, a particle which, under the conditions employed in these studies, is phagocytosed predominantly by MFR (6). These data strongly suggest that the MFR or a similar receptor mediates uptake of unopsonized *P. aeruginosa*.

Discussion

Macrophage phagocytic receptors for both opsonized and unopsonized particles have been characterized previously (1, 2).

Table III. Phagocytosis of *P. aeruginosa* and EIgG by Macrophages Plated on Fc Fragment-coated Coverslips

Substrate	Phagocytosis			
	<i>P. aeruginosa</i> *			EIgG†
	<6	6-19	>19	
Control (BSA)	6	48	46	7.7
Anti-BSA (0.6 mg/ml)	57	42	1	0.1
Fc fragments of anti-BSA	46	49	5	0.7

* Percentage of macrophages with indicated number of bacteria ingested.

† Average number of particles ingested per macrophage.

Table IV. Maturation of Fc Receptor Function during In Vitro Cultivation of Human Monocytes

Duration of culture	Phagocytosis of EIgG	
	Particles/macrophage	Percentage of macrophages with ingested particles
d		
0	2.4	89
1	4.6	93
2	7.1	100
3	8.0	98
4	4.8	84

Table V. The Effect of Mannan on Phagocytosis of *P. aeruginosa*, IgG, and Zymosan by Macrophages

Condition	Phagocytosis				
	<i>P. aeruginosa</i> *			IgG [‡]	Zymosan [‡]
	<6	6–19	>19		
Control [§]	15	66	19	9.3	3.9
Mannan (10 mg/ml)	81	19	0	4.2	0.2
Mannan (1 mg/ml)	64	36	0	5.8	0.3
Mannan (100 µg/ml)	13	74	13	5.4	0.8
Mannan, derivatized [†]	56	42	2	6.8	1.2

* Percentage of macrophages with the indicated number of bacteria ingested.

[‡] Average number of particles ingested per macrophage.

[§] Macrophages plated on coverslip derivatized with 10 mg/ml BSA.

^{||} Mannan added to phagocytosis medium at indicated final concentration.

[†] Macrophages plated on coverslip derivatized with 50 mg/ml mannan.

In this paper, we have described the functional characteristics of human macrophage receptors for phagocytosis of unopsonized *P. aeruginosa*. Although *P. aeruginosa* is also phagocytosed nonopsonically by human PMN (3), pulmonary alveolar macrophages (5), and mouse peritoneal macrophages (Speert and Silverstein, unpublished observations), the studies reported here are confined to human monocyte-derived macrophages.

The macrophage receptors for mannose-type oligosaccharides and the macrophage surface molecules that mediate the uptake of unopsonized *P. aeruginosa* share several functional characteristics (Table IX). Both receptors are under maturational control; they are absent from peripheral blood monocytes (19) and do not become functionally competent until after at least 48 h of in vitro cultivation (6). Phagocytosis mediated by both receptors is inhibited in the presence of mannose (6), and both are down-modulated when macrophages are allowed to spread on surfaces derivatized with mannan or with IgG. Finally, both receptors are divalent cation-dependent, trypsin-sensitive and capable of promoting phagocytosis in the absence of phorbol esters or fibronectin if the macrophages mature in suspension in teflon vessels (15). These characteristics clearly differentiate between unopsonized *P. ae-*

ruginosa's receptors, those of C3bi, and those of the Fc portion of IgG (1, 2).

We found that macrophages plated on IgG-coated surfaces lost the capacity to phagocytose via both Fc receptors and MFR (Table II). *P. aeruginosa* phagocytosis was impaired when macrophages were allowed to spread on either mannan (Table V) or IgG-coated (Table II) surfaces. A satisfactory explanation for this phenomenon of co-down-modulation has not yet been provided. Nose and Wigzell (20) reported that IgG, which was depleted of asparagine-linked carbohydrates, lost the capacity to interact with macrophage Fc receptors. It is therefore possible that carbohydrates on IgG are of critical importance in the ligation of both Fc receptors and MFR.

In addition to their role in the phagocytosis of glycosylated macromolecules (21), MFRs play a key role in the ingestion of unopsonized zymosan (6, 15). Under the conditions described in this paper, unopsonized zymosan is phagocytosed by macrophages (6), but not by peripheral blood monocytes (6) or by unstimulated PMNs (unpublished observation); of these three types of human phagocytic cells, only macrophages possess MFR (19). Serum opsonin-independent phagocytosis of certain gram-negative bacterial species by PMNs is inhibited by mannose (3, 22–25). In these situations, the inhibitory effects of mannose are correlated with the presence of mannose-binding adhesins on *Escherichia coli* (25) and with hydrophobic pili on *P. aeruginosa* (4). It is possible that both *E. coli* and *P. aeruginosa* possess mannose-specific lectins on their outer membranes that interact with neutrophil carbohydrate residues. The presence of such a lectin-carbohydrate interaction has been proposed (27, 28). Recently, three human neutrophil membrane glycoproteins with molecular masses of 70–80 and 150 kD have been shown to bind type 1 *E. coli* pili in a mannose-sensitive fashion (29). Thus the mannose-sensitive phagocytosis of bacteria by neutrophils may involve bacterial lectins; conversely, mannose-sensitive phagocytosis of bacteria by macrophages appears to involve lectin-like receptors and bacterial carbohydrates. It is possible that mannose-sensitive phagocytosis of bacteria by macrophages may involve two separate receptor-ligand interactions which are mediated by either bacterial or macrophage lectins. The *P. aeruginosa* strain studied by us has surface-exposed mannose residues (mannose-sensitive agglutination by Con A) but it lacks mannose-sensitive lectin activity (inability to agglutinate human red blood cells) (unpublished observation). The mannose-sensitive

Table VI. The Effect of Divalent Cations on Phagocytosis of Different Particles

Condition	Phagocytosis					Binding EC3bi†
	<i>P. aeruginosa</i> *			EIgG‡	Zymosan‡	
	<6	6–19	>19			
Control (RPMI with Ca ²⁺ and Mg ²⁺)	16	64	20	8.8	6.5	4.6
RPMI + EGTA (2 mM)	71	29	0	8.9	0.9	0.3
RPMI + EDTA (2 mM)	97	3	0	6.3	0.6	0.8
GVB [–] (divalent cation-free)	97	3	0	6.9	0.1	0.0
GVB [–] + Mg ²⁺ (0.5 mM)	86	14	0	6.3	1.2	3.6
GVB [–] + Ca ²⁺ (0.5 mM)	70	30	0	6.4	1.9	1.9
GVB [–] + Mg ²⁺ + Ca ²⁺	71	28	1	5.7	2.4	4.7

* Percentage of macrophages with the indicated number of bacteria ingested. [‡] Average number of particles per macrophage.

Table VII. The Effect of Enzyme Treatment of Macrophages on Phagocytosis or Binding of Different Particles

Condition	Phagocytosis			ElgG [‡]	Zymosan [‡]	Binding
	<i>P. aeruginosa</i> [*]					EC3bi [‡]
	<6	6–19	>19			
Control	1	22	77	9.8	4.8	6.4
α Galactosidase (10 μ g/ml)	0	17	83	7.6	4.7	—
α Mannosidase (100 μ g/ml)	3	23	74	11.2	4.8	—
Trypsin (100 μ g/ml)	22	57	21	11.9	0.6	1.0
Trypsin (500 μ g/ml)	40	47	13	8.5	0.1	—
Pepsin (100 μ g/ml)	1	16	83	8.6	3.0	—
Protease (10 μ g/ml)	5	25	70	8.4	2.3	—
Protease (100 μ g/ml)	20	57	23	10.4	0.3	—
Neuraminidase (10 μ g/ml)	8	26	66	15.9	4.5	—
Neuraminidase (100 μ g/ml)	3	31	66	15.5	3.5	—
Papain (10 μ g/ml)	23	45	32	12.9	3.1	—
Papain (100 μ g/ml)	30	50	20	18.2	3.1	—

* Percentage of macrophages with indicated number of bacteria ingested. [‡] Average number of particles per macrophage.

phagocytosis of *P. aeruginosa* by macrophages is therefore most likely mediated by macrophage rather than by bacterial lectins.

While our studies show that the MFR is necessary for the recognition of unopsonized *P. aeruginosa*, they do not rule out the participation of other M ϕ receptors. Other microbes (*Histoplasma capsulatum* (30) and *E. coli* (18) have been shown to be recognized nonopsonically by the LFA-1, CR3, p150,95 family of adhesion-promoting glycoprotein receptors; our data suggest that these receptors may also participate in the recognition of *P. aeruginosa*. There was marked inhibition of *Pseudomonas* phagocytosis when M ϕ were plated on surfaces coated with MAb IB4, which recognizes all three members of this glycoprotein family (Table I). Since the inhibition caused by IB4 was far greater than that caused by other IgGs, it is unlikely that the Fc domain of IB4 accounts for all the inhibition. Rather, down-modulation of LFA-1, CR3, and p150,95 may

decrease phagocytosis of *P. aeruginosa* by removing receptors that function in cooperation with the MFR. Other investigators (17) have demonstrated that CR3 has lectin-like properties and is capable of mediating ingestion of unopsonized zymosan. They further described two separate epitopes of CR3, one that mediates binding of C3bi-coated particles and another that mediates ingestion of zymosan. CR3 could therefore mediate phagocytosis of unopsonized *P. aeruginosa* by interacting with bacterial surface-exposed glycoconjugates. Ingestion but not attachment of C3bi-coated particles by CR3 is under maturational control, first occurring after the cells have been in culture for more than 48 h (31). This further suggests a role for CR3 in the recognition of unopsonized *P. aeruginosa*, phagocytosis of which was not observed until monocytes had been in cultures for at least 48 h.

Nonopsonic phagocytosis of *P. aeruginosa* by human macrophages may play an important role in normal host defense of the lung. Macrophages are part of the first line of lung defense and must often function in a milieu where there are low levels of both complement and Ig; phagocytosis of some strains of *P. aeruginosa* by macrophages is, in fact, inhibited by serum (7,

Table VIII. The Effect of Different Sugars on Phagocytosis of *P. aeruginosa* and Zymosan by Macrophages

Sugar*	Phagocytosis			Zymosan†
	<i>P. aeruginosa</i> ‡			
	<6	6-19	>19	
Control	10	52	38	7.71
D-mannose	19	67	13	1.82
L-mannose	20	42	38	6.04
D-fucose	16	63	22	5.15
L-fucose	40	52	8	2.12
α MM	34	58	8	1.79
D-glucose	12	61	27	5.62

* All sugars were used at a final concentration of 10 mg/ml and were present during the phagocytosis assay.

[‡] Percentage of macrophages with the indicated number of bacteria ingested.

[§] Average number of particles ingested per macrophage.

Table IX. Characteristics of Different Phagocytic Receptors on Monocyte-Derived Macrophages

Characteristic	Receptor			
	FcR	CR3	Mannose	<i>Pseudomonas</i>
Appears during in vitro maturation	No	No	Yes \geq 3 d	Yes \geq 2 d
Inhibited by mannan in suspension	Yes	ND	Yes	Yes
Down-modulated by mannan	Yes	ND	Yes	Yes
Divalent cation dependent	No	Yes	Yes	Yes
Trypsin sensitive	No	Yes	Yes	Yes
Down-modulated by IgG	Yes	No	Yes	Yes

8, and Speert and Thorson, unpublished observation). Subversion of this nonopsonic phagocytic process by bacterial or host products might then permit a relatively avirulent microorganism to gain ascendancy in the bronchopulmonary tract and to cause disease. *P. aeruginosa* exoproducts (such as the mucoid exopolysaccharide) or shed cell membrane structures (such as LPS) might compete for the pulmonary alveolar macrophage receptor for unopsonized *Pseudomonas*. Such may be the case in patients with CF since chronic bronchopulmonary infection is a hallmark of the disease (32) and bacteria in very high density are characteristically present in bronchopulmonary secretions.

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References

1. Bodmer, J. L. 1985. Membrane receptors for particles and opsonins. In *Mononuclear Phagocytes: Physiology and Pathology*. R. T. Dean and W. Jessup, editors. Elsevier, New York. 55–78.
2. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669–722.
3. Speert, D. P., F. Eftekhari, and M. L. Puterman. 1984. Nonopsonic phagocytosis of *Pseudomonas aeruginosa* strains from cystic fibrosis patients. *Infect. Immun.* 43:1006–1011.
4. Speert, D. P., B. A. Loh, D. A. Cabral, and I. E. Salit. 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. *Infect. Immun.* 53:207–212.
5. 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.
6. Speert, D. P., and S. C. Silverstein. 1985. Phagocytosis of unopsonized zymosan by human monocyte-derived macrophages: maturation and inhibition by mannan. *J. Leukocyte Biol.* 38:655–658.
7. Biggar, W. D., B. Holmes, and R. A. Good. 1971. Opsonic defect in patients with cystic fibrosis of the pancreas. *Proc. Natl. Acad. Sci. USA.* 68:1716–1719.
8. Fick, R. B., G. P. Naegel, R. A. Matthay, and H. Y. Reynolds. 1981. Cystic fibrosis *Pseudomonas* opsonins: inhibitory nature in an in vitro phagocytic assay. *J. Clin. Invest.* 68:899–914.
9. Pier, G. B., J. M. Saunders, P. Ames, M. S. Edwards, H. Auerbach, J. Goldfarb, D. P. Speert, and S. Hurwitch. 1987. Opsonophagocytic killing antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide in older noncolonized patients with cystic fibrosis. *N. Engl. J. Med.* 317:793–798.
10. Wright, S. D., P. E. Rao, W. C. van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699–5703.
11. Lanier, L. L., M. A. Arnaout, R. Schwarting, N. L. Warner, and G. D. Ross. 1985. p150/95, Third member of the LFA-1/CR3 polypeptide family identified by anti-Leu M5 monoclonal antibody. *Eur. J. Immunol.* 15:713–718.
12. Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fc receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275–3279.
13. Wright, S. D., and S. C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149–1164.
14. Michl, J., M. M. Peiczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
15. Sung, S. J., R. S. Nelson, and S. C. Silverstein. 1983. Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell Biol.* 96:160–166.
16. Sung, S. J., R. S. Nelson, and S. C. Silverstein. 1985. Mouse peritoneal macrophages plated on mannan- and horseradish peroxidase-coated substrates lose the ability to phagocytose by their Fc receptors. *J. Immunol.* 134:3712–3717.
17. Ross, G. D., J. A. Cain, and P. J. Lachmann. 1985. Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. *J. Immunol.* 134:3307–3315.
18. Wright, S. D., and M. T. C. Jong. 1986. Adhesion-promoting receptors on human macrophages recognize *E. coli* by binding to lipopolysaccharide. *J. Exp. Med.* 164:1876–1888.
19. Shepherd, V. L., E. J. Campbell, R. M. Senior, and P. D. Stahl. 1982. Characterization of the mannose/fucose receptor on human mononuclear phagocytes. *J. Reticuloendothel. Soc.* 32:423–431.
20. Nose, M., and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:6632–6636.
21. Stahl, P., P. H. Schlesinger, B. Sigardson, J. S. Rodman, and Y. C. Lee. 1980. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell.* 19:207–215.
22. Mangan, D. F., and I. S. Snyder. 1979. Mannose-sensitive interaction of *Escherichia coli* with human peripheral leukocytes in vitro. *Infect. Immun.* 26:520–527.
23. Silverblatt, F. J., J. S. Dreyer, and S. Schauer. 1979. Effect of pili on susceptibility of *Escherichia coli* to phagocytosis. *Infect. Immun.* 24:218–223.
24. Bar-Shavit, Z., R. Goldman, I. Ofek, N. Sharon, and D. Mirelman. 1980. Mannose-binding activity of *Escherichia coli*: a determinant of attachment and ingestion of the bacteria by macrophages. *Infect. Immun.* 29:417–424.
25. Pruzzo, C., E. Debbia, and G. Satta. 1982. Mannose-inhibitable adhesins and T3-T7 receptors of *Klebsiella pneumoniae* inhibit phagocytosis and intracellular killing by human polymorphonuclear leukocytes. *Infect. Immun.* 36:949–957.
26. Svanborg-Eden, C., L.-M. Bjursten, R. Hull, S. Hull, K.-E. Magnusson, Z. Moldovano, and H. Leffler. 1984. Influence of adhesins on the interaction of *Escherichia coli* with human phagocytes. *Infect. Immun.* 44:672–680.
27. Bar-Shavit, Z., I. Ofek, R. Goldman, D. Mirelman, and N. Sharon. 1977. Mannose residues on phagocytes as receptors for the attachment of *Escherichia coli* and *Salmonella typhi*. *Biochem. Biophys. Res. Commun.* 78:455–460.
28. Weir, D. M., and C. C. Blackwell. 1983. Interaction of bacteria with the immune system. *J. Clin. Lab. Immunol.* 10:1–12.
29. Rodriguez-Ortega, M., I. Ofek, and N. Sharon. 1987. Membrane glycoproteins of human polymorphonuclear leukocytes that act as receptors for mannose-specific *Escherichia coli*. *Infect. Immun.* 55:968–973.
30. Bullock, W. E., and S. D. Wright. 1987. Role of the adherence-promoting receptors, CR3, LFA-1, and p150,95, in binding of *Histoplasma capsulatum* by human macrophages. *J. Exp. Med.* 165:195–210.
31. Wright, S. D., L. S. Craigmyle, and S. C. Silverstein. 1983. Fibronectin and serum amyloid P component stimulate C3b- and C3bi-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 158:1338–1343.
32. Speert, D. P. 1985. Host defenses in patients with cystic fibrosis: modulation by *Pseudomonas aeruginosa*. *Surv. Synth. Pathol. Res.* 4:14–33.