JCI The Journal of Clinical Investigation

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J Clin Invest. 1992;90(3):1085-1092. https://doi.org/10.1172/JCI115924.

Research Article

Pseudomonas aeruginosa is an important pulmonary pathogen in cystic fibrosis, but the means by which it evades host defenses is understood poorly. Macrophages (M phi) are critical in protecting the lung and mucosal surfaces against infection and may need to perform their functions in the absence of opsonins before the evolution of an inflammatory response. The purpose of the present study was to define factors that regulate the capacity of macrophages to mediate nonopsonic phagocytosis. Phagocytosis of unopsonized P. aeruginosa by murine peritoneal and pulmonary alveolar M phi s was absolutely dependent upon the presence of glucose; only D-mannose could substitute. Glucose-dependent phagocytosis appears to be selective for P. aeruginosa by M phi s; ingestion of unopsonized zymosan, opsonized P. aeruginosa, EIgG, and E (IgM)C occurred in the presence or absence of glucose as did-ingestion of unopsonized P. aeruginosa by polymorphonuclear leukocytes. M phi binding and phagocytosis of unopsonized P. aeruginosa appeared to occur by a mechanism independent of complement receptor 3 and mannose receptors. Phagocytosis of P. aeruginosa killed by tobramycin or Formalin was glucose dependent, suggesting that the glucose exerted its effects on the M phi rather than the bacteria. The predilection of P. aeruginosa for lower airway disease in patients with cystic fibrosis might be explained in part by the unique dependency upon glucose for M phi [...]



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Phagocytosis of Unopsonized *Pseudomonas aeruginosa* by Murine Macrophages Is a Two-Step Process Requiring Glucose

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Abstract

Pseudomonas aeruginosa is an important pulmonary pathogen in cystic fibrosis, but the means by which it evades host defenses is understood poorly. Macrophages (M ϕ) are critical in protecting the lung and mucosal surfaces against infection and may need to perform their functions in the absence of opsonins before the evolution of an inflammatory response. The purpose of the present study was to define factors that regulate the capacity of macrophages to mediate nonopsonic phagocytosis. Phagocytosis of unopsonized P. aeruginosa by murine peritoneal and pulmonary alveolar M ϕ s was absolutely dependent upon the presence of glucose; only D-mannose could substitute. Glucose-dependent phagocytosis appears to be selective for P. aeruginosa by Møs; ingestion of unopsonized zymosan, opsonized P. aeruginosa, EIgG, and E(IgM)C occurred in the presence or absence of glucose as did ingestion of unopsonized P. aeruginosa by polymorphonuclear leukocytes. M ϕ binding and phagocytosis of unopsonized P. aeruginosa appeared to occur by a mechanism independent of complement receptor 3 and mannose receptors. Phagocytosis of P. aeruginosa killed by tobramycin or Formalin was glucose dependent, suggesting that the glucose exerted its effects on the M ϕ rather than the bacteria. The predilection of P. aeruginosa for lower airway disease in patients with cystic fibrosis might be explained in part by the unique dependency upon glucose for $M\phi$ phagocytosis. (J. Clin. Invest. 1992. 90:1085-1092.) Key words: cystic fibrosis • phagocyte • receptor • bacteria • infection

Introduction

Pseudomonas aeruginosa is the predominant cause of pulmonary infection in patients with cystic fibrosis, but an immunological explanation for this has not been found (1). Macrophages $(M\phi)^1$ are critical in protecting the lung and mucosal surfaces against infection and may ingest and kill potential

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J. Clin. Invest.

© The American Society for Clinical Investigation, Inc. 0021-9738/92/09/1085/08 \$2.00 Volume 90, September 1992, 1085-1092 pathogens in the absence of exogenous opsonins. Phagocytic cell dysfunction has been suggested but not demonstrated in cystic fibrosis. The purpose of the present investigations was to explore the factors that regulate phagocytosis of unopsonized *P. aeruginosa* to better understand the dynamic host-parasite relationship in cystic fibrosis lung infections.

Strains of *P. aeruginosa* from patients with cystic fibrosis are susceptible to phagocytosis by human neutrophils and M ϕ s in the absence of serum opsonins (2). Nonopsonic phagocytosis of *P. aeruginosa* is a receptor-mediated event that is facilitated by certain bacterial features; mucoid strains are relatively resistant to phagocytosis and bacterial pili appear to enhance the process (3, 4). Although the M ϕ receptor(s) mediating nonopsonic phagocytosis have been characterized functionally, their specific structures have not been identified. They share many of the characteristics of the mannosyl/fucosyl receptor yet certain features suggest that they are distinct (5).

Previous investigations of phagocytosis of unopsonized P. aeruginosa have used human monocyte-derived M ϕ s (4, 5). These studies have been frustrated by the difficulties in obtaining large numbers of cells, the biological variability among leukocyte donors, and the influences introduced by in vitro cultivation of monocytes. To gain a better understanding of receptor-mediated nonopsonic phagocytosis of P. aeruginosa, studies were initiated with murine $M\phi s$. These studies used "mature" M ϕ s and demonstrated that the receptor for unopsonized P. aeruginosa is absolutely dependent upon glucose for ingestion in distinction to the other better characterized phagocytic receptors. Glucose is present in diminishingly low concentrations in bronchial fluid (6). The propensity of P. aeruginosa to cause pulmonary infections in patients with cystic fibrosis might be explained by this unique dependency upon glucose for $M\phi$ -mediated ingestion.

Methods

Particles for phagocytosis

P. aeruginosa strain P-1 is a nonmucoid derivative of a mucoid cystic fibrosis isolate (2). It has a rough lipopolysaccharide, is susceptible to the bactericidal effect of human serum, and has from one to three polar pili per bacterium (3). It was grown overnight under static conditions in L broth (10 g tryptone [Difco Laboratories, Inc., Detroit, MI], 5 g yeast extract [Difco Laboratories, Inc.] and 10 g NaCl/liter distilled water) and frozen in aliquots at -70°C. Bacteria for each experiment were inoculated 1:100 from frozen stock in L broth and grown overnight under static conditions at 37°C. Immediately before use in phagocytosis experiments, the bacteria were gently vortexed to disrupt the pellicle and used without washing. Phagocytosis was usually assessed with unopsonized bacteria. Opsonization for some experiments was performed by tumbling the bacteria for 15 min in 1-5% heat-inactivated (56°C for 30 min) hyperimmune polyclonal rabbit serum. After opsonization, the bacteria were washed once and resuspended in PBS, pH 7.4.

February 1992.

^{1.} Abbreviations used in this paper: CR3, complement receptor 3; DMEM, Dulbecco's modified minimal essential medium; ElgG, erythrocytes opsonized with IgG; E(IgM)C, erythrocytes opsonized with IgM and complement; gHBSS, HBSS with 0.1% gelatin; $M\phi$, macrophage.

Unopsonized zymosan, erythrocytes opsonized with IgG (ElgG), and erythrocytes opsonized with IgM and complement (E(IgM)C)were prepared exactly as described (4).

Hyperimmune anti-Pseudomonas rabbit serum

Hyperimmune serum was produced by repeatedly immunizing two adult New Zealand white rabbits with Formalin-killed *P. aeruginosa* strain P-1.

Phagocytic cells

Phagocytic cells were obtained from the peritonea of mature female BALB/c mice. Leukocytes were elicited by intraperitoneal injection of 1 ml Brewer's complete thioglycollate broth (7). Cells harvested 1 d later were \sim 70% neutrophils whereas those harvested after 3-6 d were \sim 70% M ϕ s. Resident cells consisted mainly of lymphocytes and \sim 25% M ϕ s. Pulmonary alveolar M ϕ s were lavaged from freshly exsanguinated mature female BALB/c mice after intratracheal instillation of 1-2 ml PBS with 0.4% lidocaine.

Reagents

L-glucose, D- and L-mannose, α lactose, D- and L-fucose, fructose, maltose, and mannan were purchased from Sigma Chemical Co. (St. Louis, MO). D-glucose, 2-deoxy-D-glucose, sucrose, and Formalin were purchased from BDH Chemicals Ltd. (Toronto, Ontario, Canada) and tobramycin was from Eli Lilly and Company (Toronto, Ontario, Canada) β lactose was from Eastman Kodak (Rochester, NY) and pyruvate was from Gibco Laboratories (Grand Island, NY). Monoclonal antibody 5C6 is against the alpha chain of complement receptor 3 (CR3) (8).

Cell culture media were prepared by the Terry Fox Laboratory (Vancouver, BC, Canada). Leibovitz's medium (L15) contained D(+) galactose (900 mg/liter) as the carbon source. To the L15 medium was added 10 mM HEPES. Dulbecco's modified minimal essential medium (DMEM) and HBSS were prepared without glucose. RPMI 1640 medium (with 11 mM D-glucose) was prepared in the standard manner.

Phagocytosis

Mice were killed with CO₂ or by exsanguination, and peritoneal or pulmonary alveolar cells were lavaged with PBS. Leukocytes were then washed in PBS, adjusted to ~ 4×10^5 /ml in RPMI 1640, and 50 μ l was loaded onto acid-washed 11-mm diameter round glass coverslips. The coverslips were incubated at 37°C in 5% CO₂ and dipped several times in PBS to remove nonadherent cells. Peritoneal cells were used fresh and pulmonary cells were incubated overnight in 5% CO₂ before use. The coverslips were then added to duplicate 24-well plastic tissue culture plates (Becton Dickinson, Lincoln Park, NJ) with each well containing 400 μ l of the medium in which the phagocytosis assay was to be performed. The plates were incubated at room temperature in ambient CO₂ for 30 min. After this period of equilibration, 40 μ l of bacteria (prepared as described above) were added to each of the wells. The plates were incubated at 37°C at ambient CO₂ for 60 min after which the coverslips were washed by gently injecting and aspirating 1 ml PBS six times. Methanol was then added to all wells of one of the duplicate plates and the coverslips contained therein were assessed for total bacteria bound and ingested. Wells in the other plate were treated to lyse all uningested bacteria as follows: The plate was chilled on ice and 500 µl ice-cold lysozyme (5 mg/ml; Sigma Chemical Co.) in 0.25 M Tris buffer, pH 8.0, was added to each well. The plates were further incubated on ice for 5 min and then the wells were washed with PBS. Finally, bacterial spheroplasts were lysed by adding 500 μ l ice-cold distilled water for 2 min and the coverslips were washed with PBS and fixed with methanol. The coverslips were air-dried, mounted on glass microscope slides, and stained with toluidine blue (1% in 1% borax). In some experiments, the bacteria were centrifuged onto the M ϕ s for 7 min at 1,500 g at room temperature immediately after the addition of bacteria to the wells.

Intraperitoneal neutrophils were obtained from mice 1 d after thioglycollate broth injection. The cells were washed, resuspended in HBSS with 0.1% gelatin (gHBSS), and mixed in a ratio of 1 neutrophil to 10 bacteria in 4-ml polypropylene snap top tubes (Falcon Plastics, Cockeysville, MD). The mixture was tumbled end over end for 1 h at 37°C and the uningested bacteria removed by three washes in gHBSS with centrifugation (168 g). The washed neutrophils were deposited on glass slides by cytocentrifugation (cytospin 2; Shandon Southern Instruments, Inc., Sewickley, PA), air-dried overnight, and stained with crystal violet.

Phagocytosis and binding were assessed by bright-field microscopy as described previously (5). Total ingested and bound bacteria were all those associated with leukocytes. Ingested bacteria were only those associated with leukocytes after lysozyme treatment.

Electron microscopy

Transmission electron microscopy. Resident peritoneal M ϕ s (10⁷) were mixed with *P. aeruginosa* (3 × 10⁸) in a volume of 3 ml L15 medium (with or without 10 mM D-glucose) in 15-ml conical polypropylene tubes. The phagocytosis mixture was rotated for different periods of time, washed twice with PBS, and fixed with 2.5% glutaralde-hyde in 0.1 M cacodylate buffer as described (9). The specimen was dehydrated with graded concentrations of ethanol, sectioned, and viewed with an electron microscope (JEOL 100 CX; JEOL, London, UK) operating at 80 kV.

Scanning electron microscopy. Approximately 3×10^5 M ϕ s were plated on each of several glass chips and bacteria were added as described above for assessment of phagocytosis. After appropriate incubations in L15 medium (with or without 10 mM D-glucose), the monolayer was washed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Specimens were prepared as described (9) and viewed with an electron microscope (JEOL 100 CX; JEOL) with scanning attachment operating at 40 kV.

Statistics. Differences were analyzed by a two-tailed Student's t test. A P value of < 0.05 was considered statistically significant.

Results

Glucose is required for phagocytosis of unopsonized P. aeruginosa by $M\phi s$. When $M\phi s$ were incubated with unopsonized P. aeruginosa in L15, a medium that contains galactose rather than glucose, we noticed that the bacteria were bound but not ingested. Fig. 1 A demonstrates bacteria bound to thioglycollate-elicited murine peritoneal M ϕ s. After lysozyme treatment (Fig. 1 C), bacteria were only rarely seen associated with the M ϕ s. When RPMI 1640 medium (that contains glucose) was substituted for L15, phagocytosis was clearly evident (data not shown). To determine the phagocytosis-promoting factor in RPMI 1640 that was absent from L15, additional experiments were performed. When D-glucose was added, phagocytosis was enhanced in a dose-dependent fashion (Fig. 2), with 10 mM the optimum concentration. All subsequent experiments were therefore performed with 10 mM D-glucose. Glucose promoted ingestion of all 10 additional P. aeruginosa strains examined (data not shown). Bacteria that were phagocytosed in the presence of glucose (Fig. 1 B) were resistant to lysozyme-mediated lysis (Fig. 1 D). Glucose selectively promoted uptake of unopsonized P. aeruginosa. Phagocytosis of unopsonized zymosan occurred even if unopsonized P. aeruginosa was added, both in the absence (Fig. 1 E) and presence (Fig. 1 F) of 10 mM D-glucose.

Ultrastructural studies were performed to explore more fully the process by which glucose promoted nonopsonic phagocytosis. Bacteria that bound to the $M\phi$ surface in the absence of



Figure 1. The effect of glucose on phagocytosis of unopsonized Pseudomonas aeruginosa (indicated with arrowheads) and unopsonized zymosan (indicated with arrows) by thioglycollate-elicited mouse peritoneal macrophages. A and C demonstrate lack of phagocytosis of unopsonized P. aeruginosa in the absence of glucose and B and D show ingestion in the presence of 10 mM D-glucose. Treatment with lysozyme (C and D) degraded bacteria that were not within the M ϕ s, permitting differentiation between bound and ingested bacteria. Lysozyme-treated bacterial "ghosts" are indicated with open arrows in C. Incubation of both unopsonized P. aeruginosa and unopsonized zymosan with M ϕ s in the absence (E) or the presence (F) of glucose demonstrated that the former was ingested only in the presence of glucose whereas the latter was not dependent upon glucose for ingestion.



Figure 2. The effect of varying concentrations of D-glucose and D-mannose on phagocytosis of unopsonized *Pseudomonas aeruginosa* by macrophages. Each point represents the mean from three separate experiments. Note that the optimum concentration for enhancement of phagocytosis was greater for mannose (≥ 20 mM) than for glucose (≤ 10 mM).

glucose elaborated filamentous structures that were clearly seen by scanning electron microscopy (Fig. 3, A and B). These filaments appeared to be peritrichal rather than polar. The expression of these unusual structures was independent of glucose and was also observed when bacteria were plated on glass. The structures extended both among bacteria and between bacteria and M ϕ s and they appeared to be of bacterial origin. Some bound bacteria were nestled among pseudopodia (Fig. 3 C) and, after glucose was added, were observed partially obscured within collar-like M ϕ structures (Fig. 3 D).

M ϕ s were fully viable after incubation with or without Dglucose. Electron microscopic examination demonstrated that unopsonized *P. aeruginosa* entered M ϕ s in a "zippering-like" manner whereby pseudopodia engulfed the bacteria by spreading circumferentially around their surfaces. (Fig. 4). Bacteria were bound to the M ϕ surface in the absence of glucose (Fig. 4 *A*) and then were engulfed within pseudopodia after the addition of glucose (Fig. 4 *B*). Phagocytosed bacteria were subsequently observed singly (Fig. 4 *C*) or multiply (Fig. 4 *D*) within phagosomes.

The ability of other sugars to substitute for D-glucose. Dmannose was the only saccharide found that could substitute for D-glucose to enhance phagocytosis of unopsonized *P. aeruginosa* (Table I). A dose-dependent enhancing effect was seen (Fig. 2). The optimum concentration for D-mannose was ≥ 20 mM (approximately threefold higher than for D-glucose), and even at that concentration was substantially less able than glucose to augment phagocytosis (Fig. 2). Other sugars, including



Figure 3. Scanning electron micrographs demonstrating unopsonized *Pseudomonas aeruginosa* bound to the surface of murine peritoneal macrophages in the absence of glucose (A-C). The bacteria appeared to elaborate filamentous appendages connecting them to the macrophage surface (A and B). Some bound bacteria were nestled among macrophage pseudopodia (C) and were subsequently enveloped by collarlike macrophage extrusions (D)after the addition of 10 mM D-glucose. Each bar represents 1 μ m.



Figure 4. Transmission electron micrographs demonstrating phagocytosis of unopsonized *Pseudomonas aeruginosa* by murine peritoneal macrophages. Bacteria were bound to the macrophages in the absence of glucose (A). After the addition of 10 mM D-glucose, the bacteria were enveloped by pseudopodia (B) and enclosed within phagocytic vacuoles (C and D). Each bar represents 1 μ m.

2-deoxyglucose and L stereoisomers of both glucose and mannose were unable to enhance phagocytosis. Glucose was required for phagocytosis of unopsonized *P. aeruginosa* whether performed in L15, DMEM, or HBSS medium (Table I). If *P. aeruginosa* were bound to M ϕ s in the absence of glucose, phagocytosis was first seen ~ 15 min after the addition of 10 mM D-glucose (data not shown).

Functional characterization of the $M\phi$ receptors for binding and phagocytosis of unopsonized P. aeruginosa. Mannan (10 mg/ml) and antibody to CR3 (5C6, 40 μ g/ml) did not inhibit phagocytosis of P. aeruginosa although active in controls (data not shown). Therefore the murine $M\phi$ receptor for phagocytosis of unopsonized Pseudomonas appeared to be neither CR3 nor the mannosyl/fucosyl receptor.

Bacterial viability appeared to be necessary for binding to $M\phi s$ (Table II). If *P. aeruginosa* was killed by heat ($56^{\circ}C \times 30$ min) or by Formalin, neither binding nor ingestion in the presence or absence of D-glucose was observed. M ϕ viability was required for ingestion but not for binding of unopsonized *P. aeruginosa*. If the monolayer was fixed with methanol, bacteria bound to the M ϕ s in the presence or absence of glucose, but ingestion did not occur. Phagocytosis appeared to be temperature dependent, since binding was reduced and ingestion did not occur at 4°C. This inhibition of binding at 4°C could have been due to decreased bacterial motility, a characteristic of fla-

gellated *P. aeruginosa* that appears to be temperature dependent.

Role of bacterial viability in glucose-dependent phagocytosis. Experiments were performed to determine if the glucose was acting on the bacteria or the $M\phi$. Since bacterial viability appeared to be necessary for binding to occur, it was necessary to devise an experiment in which the bacteria were killed after binding to the M ϕ membrane. An agent was required that had specific effects on the bacteria without interfering with viability and function of the M ϕ . Unopsonized *P. aeruginosa* were bound to M ϕ s for 45 min in the absence of D-glucose. Tobramycin (20 μ g/ml) was then added for 60 min. This treatment killed > 99% of the bacteria. After the monolayer was washed with PBS, 10 mM D-glucose was added. Phagocytosis of unopsonized P. aeruginosa was observed whether the bacteria were killed with tobramycin (3.9 bacteria/M ϕ) or remained viable (5.3 bacteria/M ϕ). The difference in phagocytosis between these two conditions was not statistically significant (P > 0.05). However, phagocytosis of both live and killed bacteria in the presence of glucose significantly exceeded ingestion in its absence (P < 0.001 for each comparison). This experiment suggested that the glucose promoted phagocytosis by effects on the $M\phi$.

Another experiment was performed to confirm that glucose was acting on the $M\phi$ rather than the bacteria. (Table III).

Table I. Effect of Simple Sugars on Phagocytosis of ViableUnopsonized Pseudomonas aeruginosa by Macrophages

Condition	Ingested bacteria/macrophage	
	(mean/macrophage)	
Control (L15 medium)	0.6	
D-glucose (10 mM in L15)	19.6 [‡]	
L-glucose (10 mM in L15)	0	
D-mannose (10 mM in L15)	8.2 [‡]	
L-mannose (10 mM in L15)	0.6	
2-Deoxyglucose (10 mM in L15)	0.7	
α Lactose (10 mM in L15)	0	
β Lactose (10 mM in L15)	0.6	
Pyruvate (10 mM in L15)	0	
D-Fucose (10 mM in L15)	0	
L-fucose (10 mM in L15)	0	
Fructose (10 mM in L15)	0	
Maltose (10 mM in L15)	0	
Sucrose (10 mM in L15)	1.1	
DMEM without glucose	2.4	
DMEM + 10 mM D-glucose	17.1 [§]	
gHBSS without glucose	0.2	
gHBSS + 10 mM D-glucose	20.0 [§]	

* Macrophages were incubated with bacteria for 60 min and washed, after which uningested bacteria were lysed with lysozyme. Data reported in this and subsequent tables are means. Wide standard deviations existed and represent the large difference in degree of phagocytosis among different macrophages. [‡] Different from number of bacteria ingested in L15 medium control (P < 0.001). [§] Different from number of bacteria ingested in simultaneous medium control without glucose added (P < 0.001).

Bacteria were killed with Formalin, washed, and then centrifuged onto coverslips upon which $M\phi$ s had been plated. Phagocytosis of both live and Formalin-killed bacteria was substantially enhanced in the presence of glucose. Whereas live bacteria were well ingested with or without centrifugation, Formalin-killed bacteria were ingested only when centrifuged onto the $M\phi$ s.

Table II. Effect of Macrophage and Bacterial Viabilityand of Temperature on Binding and Phagocytosisof Unopsonized Pseudomonas aeruginosa

	Bacteria/macrophage			
Condition	Without added glucose		10 mM D-glucose	
	Ingested*	Bound and ingested*	Ingested	Bound and ingested
Control	0.4	9.6	10.2	13.0
Heat-killed bacteria				
(56°C ×30 min)	0	0	0	0
Formalin-killed bacteria	0.3	0.2	0.3	0.5
Methanol-fixed				
macrophages	0	18.5	0	19.8
Incubation at 4°C	0.7	2.9	0.4	5.2

* Ingested and bound and ingested were determined as described in Table I.

Table III. Bacterial Viability Is not Required for Glucose-induced
Phagocytosis of Pseudomonas aeruginosa

Bacterial viability	Centrifuged onto macrophages [‡]	Ingested bacteria/macrophage*		
		Without added glucose	10 mM D-glucose	
Viable	No	0	13.5 [§]	
Viable	Yes	0.4	25.1 [§]	
Formalin-killed	No	2.5	1.8	
Formalin-killed	Yes	1.6	7.1 [§]	

* Macrophages were incubated with bacteria for 60 min and washed, after which uningested bacteria were lysed with lysozyme. [‡] Bacteria were forced onto the monolayer by centrifugation at 1,500 g for 7 min or were allowed to contact the monolayer without centrifugation. [§] Different from number of bacteria ingested in absence of added glucose (P < 0.001).

Finally, *P. aeruginosa* were grown in L-broth supplemented with 10 mM D-glucose. When the bacteria were added to the L15 phagocytosis buffer, the glucose was diluted to 0.5 mM and phagocytosis was not facilitated (data not shown). This further suggested that the glucose was not inducing elaboration of a phagocytosis-promoting ligand on the bacteria.

Effect of D-glucose on phagocytosis of unopsonized Pseudomonas aeruginosa by different phagocytic cells. Resident and thioglycollate-elicited peritoneal and pulmonary alveolar M ϕ s from BALB/c mice ingested unopsonized P. aeruginosa to a similar extent, but both required D-glucose (Table IV). The same was true for biogel-elicited peritoneal and bone marrowderived M ϕ s from BALB/c mice and peritoneal M ϕ s from C57 mice (data not shown). Although those diverse M ϕ phenotypes were all dependent upon glucose for phagocytosis, murine neutrophils were able to bind and ingest unopsonized P. aeruginosa in the absence of added sugar (Table IV).

Enhancement of phagocytosis by D-glucose was specific for P. aeruginosa. Unopsonized P. aeruginosa was the only particle tested that was not ingested by peritoneal M ϕ s in the absence of D-glucose (Table V). Phagocytosis of unopsonized zymosan E(IgM)Cs and EIgGs was equivalent in the absence

Table IV. Glucose Is Required for Phagocytosis of Unopsonized Pseudomonas aeruginosa by Macrophages but not by Neutrophils

	Ingested bacteria/macrophage*		
Phagocytic cell	Without added glucose	10 mM D-glucose	
Resident peritoneal			
macrophage	0.6	11.7 [‡]	
Thioglycollate-elicited			
peritoneal macrophage	2.2	10.9 [‡]	
Pulmonary alveolar			
macrophage	0	7.6 [‡]	
Thioglycollate-elicited			
peritoneal neutrophil	7.1	7.2	

* Macrophages were incubated with bacteria for 60 min and washed, after which uningested bacteria were lysed with lysozyme. [‡] Different from number of bacteria ingested in the absence of added glucose (P < 0.001).

Table V. Glucose Is Required Selectively for Phagocytosis of Pseudomonas aeruginosa

	Particles/macrophage*		
Particle	Without added glucose	10 mM D-glucose	
Unopsonized P. aeruginosa	0	10.7 [‡]	
Opsonized P. aeruginosa	8.2	18.1 [‡]	
Unopsonized zymosan	7.7	7.0	
E IgG	4.6	5.3	
E(IgM)C	2.4	3.5	

* Data represent mean number of particles ingested per macrophage except for E(IgM)C in which particles bound per macrophage was determined. [‡] Different from number of bacteria ingested in the absence of added glucose (P < 0.001).

and presence of the sugar. When *P. aeruginosa* was opsonized with polyclonal rabbit serum, phagocytosis occurred in the presence or absence of glucose. The enhanced phagocytosis in the presence of glucose could have reflected the combined activities of both opsonic and nonopsonic receptors.

Discussion

Although the means by which glucose enhances phagocytosis of unopsonized *P. aeruginosa* remain explained incompletely, it appears to do so by acting on the M ϕ rather than the bacteria. This conclusion is based on the following evidence: (*a*) although M ϕ s required glucose for phagocytosis of *Pseudomonas*, PMNs were fully competent to ingest in the absence of glucose; (*b*) if bacteria killed by tobramycin or heat were bound to M ϕ s, glucose facilitated their ingestion; (*c*) mannose was the only sugar able to substitute for glucose in promoting phagocytosis; this is consistent with previous observations in which these were the only two monosaccharides able to reverse the inhibitory effects of 2-deoxyglucose (10), suggesting a specific energy-requiring event; and (*d*) growth of *P. aeruginosa* in a glucose-replete medium did not facilitate phagocytosis.

Observations from these studies provide novel insights into the mechanism by which $M\phi$ ingest unopsonized *P. aerugin*osa. Phagocytosis of *Pseudomonas* appears to be unique in its dependence upon glucose, which was required for ingestion by $M\phi$ s but not by neutrophils. A clear two-step process was observed in which binding occurred independent of glucose or $M\phi$ viability, and ingestion was dependent upon glucose but did not require bacterial viability.

Glucose-dependent ingestion appears to be specific for *P. aeruginosa* and was observed with 10 different bacterial isolates (data not shown). Other Gram-negative bacterial species (including *Escherichia coli* and *Salmonella typhimurium*) were ingested equally well in the absence and presence of glucose (data not shown). We are currently attempting to identify and purify the putative bacterial ligand, which might be one of various mannose- or galactose-specific *P. aeruginosa* lectins (11). It is possible that the nonopsonic receptor is similar to the glycolipids that bind specifically to both *P. aeruginosa* and *Pseudomonas cepacia* (12).

CR3 possesses some of the characteristics of the receptormediated phagocytic processes described in this paper. Although CR3 is competent constitutively to bind particles coated with iC3b, ingestion only occurs if the M ϕ s are exposed to agents such as phorbol esters or fibronectin (13, 14). Whereas such observations indicate two steps in CR3-mediated ingestion, both appear to be mediated by a single type of receptor. Unlike phagocytosis by CR3, ingestion of unopsonized *P. aeruginosa* was unaffected by phorbol myristate acetate in the absence or presence of glucose or by antibody to CR3 (data not shown). Furthermore, resident peritoneal M ϕ s were able constitutively to ingest unopsonized *P. aeruginosa*, whereas complement-coated particles are bound but not ingested under the same conditions.

A role for glucose in receptor-mediated phagocytosis has been investigated previously (10). Studies were performed in which 2-deoxyglucose interfered with ingestion but not binding by both Fc and complement receptors (10), unlike observations from the present studies in which these receptors performed normally in the absence of glucose. No inhibition of ingestion of two unopsonized particles (zymosan and latex) was observed. Michl and co-workers (15) suggested that the 2-deoxyglucose exerted its inhibitory effects on the M ϕ rather than the phagocytosed particle but were unable to demonstrate its mechanism of action. A further series of investigations by Sung and Silverstein (16) suggested that 2-deoxyglucose did not inhibit phagocytosis by abrogating glycosylation, protein synthesis, or energy stores.

Phagocytosis is an active process dependent upon recruitment of specific receptors to the plasma membrane. The mechanism by which glucose promotes this process with unopsonized *P. aeruginosa* remains to be determined. Glycolysis provides the energy required for phagocytosis (17), and glucose may serve as the necessary substrate. M ϕ hexokinase activity is very high relative to other cell types, suggesting that glucose is an important metabolic fuel (18). Alternatively, glycosylation of an essential membrane protein or lipid may be required for ingestion of unopsonized *P. aeruginosa*. Such a modification could be required to alter the functional state of a specific receptor or to permit transduction of a signal for facilitating the ingestion of a bound particle.

P. aeruginosa, which bound to $M\phi$ s in the absence of glucose, elaborated a network of filaments emanating in a peritrichal array. This was an unexpected observation and one that could not have been predicted from previous published observations. These may be novel structures, since the other major appendages (pili and flagella) arise only from the bacterial poles (19). Similar appendages have been demonstrated on other Gram-negative bacteria when they adhere to eukaryotic surfaces (20, 21). In fact, contact with specific types of surfaces induces *Salmonella* spp. to produce new major cell surface components (22). These structures were elaborated in the presence and absence of glucose and therefore probably did not act to inhibit phagocytosis.

Previous studies on nonopsonic phagocytosis of *P. aeruginosa* have been frustrated by an inability to differentiate between bound and ingested bacteria. The observation that lysozyme could disrupt uningested bacteria was unexpected since most strains of *Pseudomonas* are lysozyme resistant. The susceptibility of strain P-1 (used in our studies) to lysozyme is probably due to the fact that it is a clinical isolate with a rough LPS (23), whereas most strains of *P. aeruginosa* used in laboratory investigations are LPS-smooth. This susceptibility to lysozyme-mediated lysis permitted us to clearly separate binding from ingestion. Furthermore, the unique inability of M ϕ s to

ingest unopsonized *P. aeruginosa* in the absence of glucose provided an opportunity to characterize functionally the two discrete stages of binding and ingestion. Aided by these observations, we intend to characterize the receptors mediating binding and ingestion of unopsonized *P. aeruginosa* and to develop reagents capable of blocking these processes. With such reagents in hand, it should be possible to gain a better understanding of the modulation of nonopsonic receptor-mediated processes and to determine the role that these receptors might play in normal host defense against infection.

Nonopsonic phagocytosis by $M\phi$ s may be critically important in defense of the lower respiratory tract against infection before the initiation of an inflammatory response with the attendant influx of neutrophils, complement, and immunoglobulin. Bronchial fluid has diminishingly low levels of glucose present under normal conditions (6). The predilection of *P. aeruginosa* for lower airway disease in patients with cystic fibrosis might be explained in part by the unique dependency upon glucose for $M\phi$ -mediated ingestion of this particular bacterial species.

Acknowledgments

The excellent technical assistance of Ms. Mary Bergin and Ms. Lisa Thorson is gratefully acknowledged. Dr. Andrew McWilliam suggested the method for lysozyme lysis of uningested bacteria, without which this study would not have been possible.

This work was supported by grants from the Medical Research Council of Canada, the Medical Research Council (UK), the Canadian Cystic Fibrosis Foundation, the Network Centres of Excellence (Canadian Bacterial Diseases Network), and a Detweiler Travel Fellowship from the Royal College of Physicians and Surgeons of Canada. Dr. Speert is a scholar of the Canadian Cystic Fibrosis Foundation.

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