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Pseudomonas aeruginosa Cytotoxin: Periplasmic Localization and Inhibition of Macrophages

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Pseudomonas aeruginosa **cytotoxin has been isolated previously from cell autolysates. Both purified cytotoxin and periplasmic contents (osmotic shock fluid) cross-reacted on Western immunoblots with antibodies specific for cytotoxin. In addition, both preparations caused a significant reduction in antibody-mediated phagocytosis of *P. aeruginosa* M2 by mouse macrophage cell line $P388_{D1}$. Phagocytosis was restored in each case on preincubation of cytotoxin or periplasmic contents with anti-cytotoxin serum. Both cytotoxin and periplasmic contents caused depolarization of the $P388_{D1}$ cell membrane, as demonstrated with a polarization-sensitive fluorescent probe. Similar correlations were not observed for other *P. aeruginosa* cell fractions or for osmotic shock fluid from *Escherichia coli* C600. These data indicate that *P. aeruginosa* cytotoxin is localized in the periplasm and has the potential to inhibit macrophage-mediated phagocytosis, possibly by perturbing ion gradients across the macrophage plasma membrane.**

Pseudomonas aeruginosa is an opportunistic pathogen that is capable of producing life-threatening disease in immunocompromised individuals. Those who are especially at risk include patients with severe burns, cancer, diabetes, or cystic fibrosis. In those with cystic fibrosis, *P. aeruginosa* can cause persistent lung infections, indicating that the host immune system is incapable of clearing the bacteria. As macrophages represent one of the primary lines of defense against infections (6, 22), it has been suggested that these phagocytes may not be functioning correctly in the lungs of patients with cystic fibrosis (27).

One of the ways in which *P. aeruginosa* may protect itself from such basic host defenses is through production of a cytotoxin. *P. aeruginosa* cytotoxin, previously named leukocidin, has been isolated from autolysates of *P. aeruginosa* cells and appears to be associated with all isolates of *P. aeruginosa* (3). It inactivates eucaryotic cells by forming lesions or pores in the membrane of target cells of the immune system (16). This results in increased plasma membrane permeability to small molecules and ions (25). Such intoxication has been documented in granulocytes (2), endothelial cells (29), Ehrlich ascites tumor cells (16), and human leukemic cells (24). In the case of granulocytes, treatment with the cytotoxin causes an inhibition of the ability of the granulocytes to kill *P. aeruginosa* cells (2). The present study was designed to determine the bacterial cellular localization of cytotoxin and to examine its effect on macrophages. Toward this end, various bacterial cell compartments were tested for the presence of cytotoxin, and osmotic shock fluid (periplasmic contents) and a purified preparation of cytotoxin were observed for their interaction with mouse macrophage cell line $P388_{D1}$. Results of previous studies have indicated that this cell line is an appropriate model for unelicited mouse peritoneal macrophages and cultured human peripheral blood monocytes in the assessment of opsonized phagocytosis of *P. aeruginosa* (4).

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MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO1 strain H103 (19) was used as a standard strain for the isolation of osmotic shock fluid, growth supernatant, and inner and outer membranes. Other strains used were *P. aeruginosa* M2 (28) and 158 (15) and *Escherichia coli* C600 (1). All cultures were maintained on 1% proteose peptone no. 2 medium (Difco Laboratories, Detroit, Mich.). Prior to use in the phagocytosis assay, strain M2 was grown for 16 h in BM2-glucose broth (8). Cells were washed and suspended to a concentration of 10^9/ml in phosphate-buffered saline (PBS).

Sample preparation. *P. aeruginosa* cytotoxin (15); osmotic shock fluid, which was prepared by the Mg^{2+} and freeze-thaw method (12, 21); and inner and outer membranes (10) were prepared as described previously. These preparations contained, respectively, ≤0.01, 0.15, 0.24, and 0.43 μg of lipopolysaccharide (LPS) per μg of protein. Cytotoxin was obtained from strain 158, while osmotic shock fluids and membranes were prepared from strain H103. *E. coli* osmotic shock fluid was isolated from strain C600 by the method of Neu and Heppel (18). Growth supernatant samples were prepared from *P. aeruginosa* H103 cells that were grown for 20 h in shaken BM2-glucose broth (8). Growth supernatant was decanted after removal of cells by centrifugation at 13,000 × *g* for 10 min at 4°C. The supernatant was lyophilized and suspended in 10 mM Tris hydrochloride (pH 7.4) at a concentration that was 150-fold higher than the original concentration. Extensive dialysis was performed against 10 mM Tris hydrochloride buffer (pH 7.4) to remove low-molecular-weight substances. Samples were stored at −70°C until use.

Maintenance and growth of macrophage cell line $P388_{D1}$. Mouse macrophage cell line $P388_{D1}$ was maintained at 37°C in 10% CO_2 in Nunc flat-bottomed flasks (GIBCO, Burlington, Ontario) by using RPMI 1640 medium (GIBCO) supplemented with 44 mM sodium bicarbonate (Fisher Scientific Co., Vancouver, British Columbia, Canada)–10% fetal bovine serum (GIBCO)–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Terochem Laboratories, Vancouver, British Columbia, Canada)–0.4% (vol/vol) 2-mercaptoethanol (Bio-Rad Laboratories, Mississauga, On-

tario, Canada)–2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.)–40 U of penicillin per ml–40 μg of streptomycin (pH 7.2; GIBCO) per ml.

Phagocytosis assay. The visual assay for phagocytosis of *P. aeruginosa* M2 was performed as described previously (4). Briefly, 1 ml of RPMI 1640 medium without fetal bovine serum or 2-mercaptoethanol was added to a washed, cultured macrophage monolayer to give a final concentration of 10^6 cells per ml. To assess the inhibition of phagocytosis, PBS, cytotoxin, or one of the subcellular fractions was added to the macrophage monolayer 15 min prior to the addition of antibody and bacteria. Inhibitors were added in 100-μl volumes (13 μg of cytotoxin, 500 μg of osmotic shock fluid [from 1×10^{10} bacterial cells], 400 μg of growth supernatant [1×10^{11} cells], or 500 μg of inner or outer membrane [2×10^{11} cells]). Anti-protein F monoclonal antibody MA5-8 (titer, 10^8) was used in all visual phagocytosis assays at a volume of 30 μl per assay and was prepared as described previously (17). Rabbit anti-cytotoxin serum was prepared by the method of Harboe and Ingild (11) as described by Baltch et al. (3) and was used at 13 μl per assay. A total of 1 μl of this antiserum was shown to prevent 1 μg of cytotoxin from inducing the swelling of human granulocytes. The cytotoxin preparation used to raise the antiserum was pure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunodiffusion, and enzymatic methods for determining the presence of other known pseudomonal components (16). If used, antiserum was preincubated with inhibitor for 5 min before it was added. Bacterial cells were used in the assay at a *P. aeruginosa* to macrophage ratio of 20:1. Background levels of bacterial association (obtained in the presence of PBS alone) were subtracted from the average number of bacteria associated per macrophage.

Fluorescence assay. $P388_{D1}$ cells were suspended with a pipette from the bottom of flat-bottomed flasks, centrifuged at 1,000 rpm for 10 min, suspended at a concentration of 2.5×10^6 cells per ml in fresh supplemented RPMI 1640 medium, and grown for 16 h in screw-cap Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) jars (Savillex, Minnetonka, Minn.) before they were assayed. After the cells were gently suspended, they were washed and suspended in an experimental solution which approximated the ion composition of RPMI 1640 medium (23). KCl was added to a final concentration of 0.3 mM to create a K^+ concentration gradient across the plasma membrane. Cells were dispensed in 1-ml assay volumes, and the carbocyanine dye 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3(5); Molecular Probes, Junction City, Oreg.] was added at a concentration of 2×10^{-6} M. Carbocyanine dyes are lipophilic probes which are highly fluorescent in aqueous environments and minimally fluorescent in the hydrophobic environment of the membrane (23). When ion fluxes were generated across the membrane, the probe was either shunted out of the membrane to become more fluorescent (on depolarization, this decreases the electrical potential gradients across the plasma membrane) or more probe was inserted into the membrane to become less fluorescent (on hyperpolarization). To test the effect of cytotoxin and osmotic shock fluids on the plasma membrane of macrophages, various concentrations were added to cells equilibrated with diS-C_3(5) and the change in fluorescence (excitation at 620 nm; emission at 670 nm) was measured in a spectrofluorimeter (650-10S; The Perkin-Elmer Corp., Norwalk, Conn.). All samples were added in 100-μl portions (volumes equalized with PBS [pH 7.2]). Valinomycin (Sigma) was used at a concentration of 2×10^{-6} M and was a positive control for depolarization. Rates of depolarization were measured from the maximal slopes obtained from the spectrofluorimeter trace. Maximal rates were always attained within 1 min of the addition of stimulus. Data were obtained from independent experiments on three separate days.

Characterization of samples. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) and Western immunoblotting techniques (17) were performed as described previously. Samples were run in the presence of 5% 2-mercaptoethanol.

The LPS content of the samples was determined by the established 2-keto-3-deoxyoctonate assay (14).

RESULTS

Cellular localization of cytotoxin. Cytotoxin had been previously isolated from cellular autolysates. These were the supernatant fractions of stationary-phase *P. aeruginosa* cells which had been suspended in PBS and incubated for 56 h at 37°C (15, 26). A variety of experiments were performed to obtain the release of cytotoxin, but these methods did not definitively establish the cellular compartment with which cytotoxin was associated, although they did establish that cytotoxin is cell associated and can be released by various lysis techniques (26). To determine the cellular localization of cytotoxin in *P. aeruginosa*, several cell compartments were tested for the presence of cytotoxin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting techniques (Fig. 1). While rabbit anti-cytotoxin serum reacted in Western immunoblots with heavily overloaded inner and outer membrane protein preparations, no discernible bands were seen at the molecular weight of cytotoxin (Fig. 1B, lanes 4 and 5). Similarly, in growth supernatants that were concentrated 150-fold, the only immunolabeled band in anti-cytotoxin blots was at approximately 56 kilodaltons (kDa) (Fig. 1B, lane 3). These membrane and growth supernatant proteins cross-reacted strongly with anti-exoenzyme S serum (a generous gift from T. I. Nicas).

In contrast, osmotic shock fluids that were prepared by the Mg^{2+} and freeze-thaw method of Hoshino and Kageyama (12) (which is the preferred method for the isolation of the periplasmic contents of *P. aeruginosa* [21]) contained a polypeptide band of approximately 28 kDa which reacted relatively strongly with anti-cytotoxin serum on Western immunoblots (Fig. 1B, lane 2; a minor species of 27 kDa was also evident on some blots and may have been a proteolytic breakdown product of cytotoxin). The presence of a 28-kDa cross-reactive band in the periplasmic fraction was reproducibly obtained with eight separate preparations of lysate obtained by osmotic shock. No cross-reactive bands were seen on the anti-exoenzyme S blot.

Cytotoxin inhibition of phagocytosis. In previous investigations, cytotoxin had been shown to cause swelling and lysis of polymorphonuclear leukocytes (2, 25), pulmonary artery endothelial cells (29), and Ehrlich ascites tumor cells (16). In the case of polymorphonuclear leukocytes, cytotoxin was demonstrated to inhibit the bactericidal activity of these cells against *P. aeruginosa* (2). To confirm that osmotic shock fluid contained a cytotoxin, we determined the effects of sublethal concentrations of cytotoxin and osmotic shock fluid on the ability of the macrophage cell line $P388_{D1}$ to phagocytose *P. aeruginosa* cells.

Purified cytotoxin was used in the assay at 13 μg/ml. This concentration did not appear to alter significantly the viability of the macrophages during the assay, as assessed by the ability of macrophages to adhere to glass surfaces, although

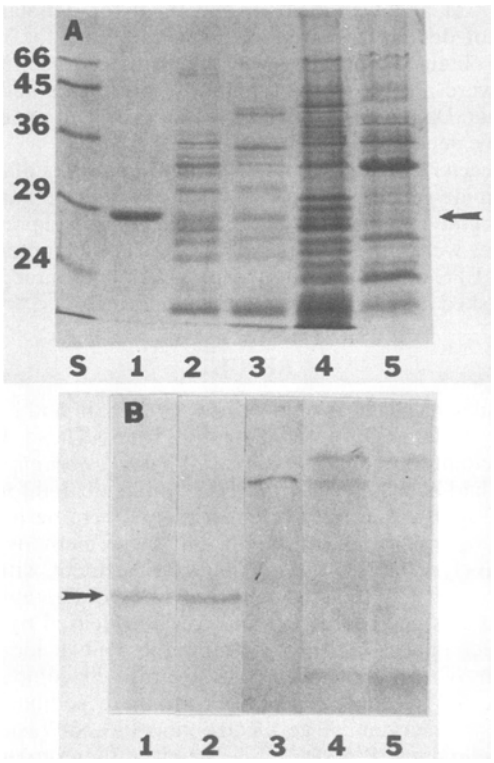

FIG. 1. Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel (A) and corresponding Western immunoblots (B) probed with anti-cytotoxin serum. Lanes 1, Purified cytotoxin; lanes 2, osmotic shock fluid method; lanes 3, growth supernatant; lanes 4, inner membrane; lanes 5, outer membrane. Positions of molecular weight standards (lane S) are indicated on the left of the gel in panel A. The positions of the 28-kDa cytotoxin band are indicated with arrows. A total of 2.8 μg of cytotoxin and 40 μg of each of the other samples were loaded in each lane. Compared with the osmotic shock fluid, 12 times as many cells were required to produce 40 μg of growth supernatant and 20 times as many cells were required to produce 40 μg of inner and outer membranes.

minor swelling was occasionally observed. At higher concentrations, cytotoxin resulted in increasing cell damage and lysis. Lower concentrations caused reduced phagocytosis, although maximal effects were seen at 13 μg/ml. At a bacteria to macrophage ratio of 20:1, 8.0 opsonized *P. aeruginosa* cells became associated per untreated macrophage (Table 1). The addition of purified cytotoxin resulted in a 95% decrease in opsonized phagocytosis. Osmotic shock fluid was also inhibitory, decreasing phagocytosis by 71%.

The addition of rabbit anti-cytotoxin restored phagocytosis by approximately 90% in each case (Table 1). This antiserum was incapable of promoting *P. aeruginosa* uptake on its own. In contrast, anti-exoenzyme S was unable to influence the inhibition of phagocytosis by osmotic shock fluid. Although the *P. aeruginosa* growth supernatant displayed a significant inhibition of phagocytosis (Table 1), the effect could be completely negated with either anti-cytotoxin or anti-exoenzyme S. Inner and outer membrane preparations increased uptake levels above those obtained in the antibody control. As a negative control, osmotic shock fluid from *E. coli* C600 was also examined in this assay. While 97% inhibition of phagocytosis was observed, the addition of anti-cytotoxin serum did not restore phagocytosis in this case (Table 1).

TABLE 1. Effect of anti-cytotoxin and anti-exoenzyme S sera on inhibition of opsonized phagocytosis of *P. aeruginosa* M2

Inhibitor	No. (%) of bacteria associated/macrophage[a]		
	No anti-cytotoxin	Anti-cytotoxin treated	Anti-exoenzyme S treated
None	8.0 (100)	—[b]	ND[c]
Cytotoxin	0.4 (5)[d]	7.0 (88)	0.4 (5)[d]
P. aeruginosa osmotic shock fluid	2.3 (29)[d]	7.3 (91)	0.3 (4)[d]
P. aeruginosa growth supernatant	2.3 (29)[d]	7.0 (88)	7.4 (93)
P. aeruginosa inner membrane	16.4 (205)	ND	ND
P. aeruginosa outer membrane	16.0 (200)	ND	ND
E. coli osmotic shock fluid	0.2 (3)[d]	1.3 (16)[d]	ND

[a] The input ratio of bacteria to macrophage cells in the assay was 20:1. Sixty macrophages per assay were assessed for the numbers of associated bacteria. Data represent the means of four to nine independent assays. Numbers in parentheses are the percent bacterial association relative to that in the absence of inhibitor.

[b] —, A single experiment with monoclonal antibody MA5-8 as the opsonin and three experiments without opsonin demonstrated that the anti-cytotoxin serum was unable to increase phagocytosis by noncytotoxin-treated macrophages.

[c] ND, Not determined.

[d] $P < 0.01$ (Student's t test) that uptake was significantly lower than that obtained in the absence of inhibitor for all assays performed.

Depolarization of the macrophage plasma membrane by cytotoxin. In previous studies it has been suggested that the mode of action of cytotoxin is to produce discrete membrane lesions (16) which allow the passage of small molecules and ions into and out of the target cell (25). It is likely that such alterations in membrane permeability cause dissipation (i.e., depolarization) of the ion gradients that are required to signal phagocytosis (e.g., a Ca^{2+} gradient [30]).

By using the polarization-sensitive fluorescent probe diS-$C_3(5)$, it was determined that cytotoxin and osmotic shock fluid do indeed produce strong depolarization of the strain $P388_{D1}$ plasma membrane (Fig. 2 and Table 2). This depolarization was similar to that caused by the positive control ionophore valinomycin in the dissipation of an imposed high K^+ concentration gradient (Fig. 2 and Table 2). The rate of depolarization increased as a function of the concentration of cytotoxin or osmotic shock fluid that was added and reached a plateau at higher concentrations (Fig. 3). As controls, the *E. coli* osmotic shock fluid (Table 2) and the concentrated growth supernatant from *P. aeruginosa* were tested and failed to produce any depolarization of the macrophage plasma membrane. LPS, when used at a concentration that was five times that found in the lysate

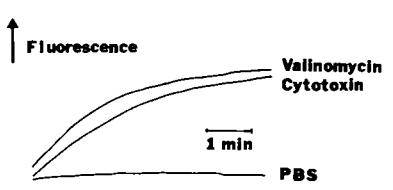

FIG. 2. Changes in diS-$C_3(5)$ fluorescence after the addition of PBS, cytotoxin (35 μg/ml), or valinomycin (2×10^{-6} M) to cells of the macrophage cell line $P388_{D1}$ that were equilibrated in the presence of 2×10^{-6} M diS-$C_3(5)$. Depolarization is indicated by an increase in fluorescence. A representative trace from three independent experiments is shown.

TABLE 2. Depolarization of the $P388_{D1}$ plasma membrane by cytotoxin, valinomycin, and osmotic shock fluid, assessed by using the fluorescent probe diS-$C_3(5)$

Addition[a]	Net fluorescence increase (arbitrary units)[b]	Initial rate of fluorescence increase (units/min)
None	0	0
Valinomycin	7.4	6.1
Cytotoxin	7.2	5.2
P. aeruginosa osmotic shock fluid	6.6	7.2
P. aeruginosa growth supernatant	0	0
P. aeruginosa LPS	0	0
E. coli osmotic shock fluid	0	0

[a] The following preparations were added, after diS-$C_3(5)$ had equilibrated across the $P388_{D1}$ plasma membrane, at the indicated final concentrations: valinomycin, 2×10^{-6} M; cytotoxin, 35 μg/ml; lysates obtained by osmotic shock, 500 μg/ml; growth supernatant, 400 μg/ml; LPS, 385 μg/ml. These concentrations were chosen to demonstrate the maximum possible effects; lower concentrations caused submaximal effects, as seen in Fig. 3.

[b] Data represent the means of three independent experiments that were performed on separate days.

obtained by the Mg^{2+} and freeze-thaw osmotic shock method (385 μg of LPS per assay), caused no measurable depolarization.

DISCUSSION

The data presented here indicate that cytotoxin is localized in the periplasmic space of *P. aeruginosa*. Thus, osmotic shock fluids reproducibly demonstrated a 28-kDa protein with antigenic cross-reactivity with purified cytotoxin. In addition, osmotic shock fluid and cytotoxin both inhibited opsonized phagocytosis of *P. aeruginosa* by macrophages, a phenomenon that could be abrogated by anti-cytotoxin serum. Furthermore, both preparations caused the formation of channels in the macrophage membrane, as assessed by the depolarization-sensitive fluorescent probe diS-$C_3(5)$.

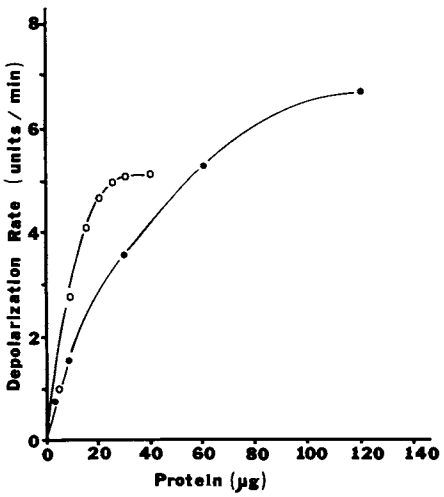

FIG. 3. Effect of increasing concentrations of cytotoxin (○) or osmotic shock fluids (●) on the rate of increase (i.e., depolarization) of diS-$C_3(5)$ fluorescence. The rates were calculated from traces such as those shown in Fig. 2 and represent the means of three different experiments.

None of the other cell fractions demonstrated similar correlations in activity with purified cytotoxin. For example, growth supernatants concentrated 150-fold contained a 56-kDa band that reacted with anti-cytotoxin serum, and this preparation could inhibit phagocytosis but was unable to depolarize the macrophage membrane. By use of anti-exoenzyme S antiserum, we were able to completely abrogate this phagocytosis inhibition and ascribe this cross-reactive band to another *P. aeruginosa* toxin, exoenzyme S. These results lead us to suggest that exoenzyme S is the major macrophage-inhibiting toxin in the growth supernatant. Presumably, other secreted pseudomonal toxins such as exoproteases, lipases, and exotoxin A do not influence macrophages in this system.

The fact that our anti-cytotoxin serum recognized the 56-kDa band may have been because of cross-reactivity of the cytotoxin with exoenzyme S or because of the presence of minor contaminating antibodies to exoenzyme S in anti-cytotoxin serum. Nevertheless, the ability of cytotoxin and osmotic shock fluid to inhibit macrophage phagocytosis was not related to the presence of exoenzyme S in these preparations, since anti-exoenzyme S serum did not react with these preparations on Western immunoblots and did not reverse the inhibitory effects of these preparations during phagocytosis of opsonized *P. aeruginosa* cells. It should be noted that in spite of the fact that the anti-cytotoxin serum recognized other bands in complex protein preparations (growth supernatant and inner and outer membranes), only the 28-kDa protein was reproducibly detected in osmotic shock fluids.

The inability of LPS to cause depolarization of the macrophage membrane in the fluorescence assay eliminated the possibility that contaminating LPS in the osmotic shock preparation was responsible for the observed results. Furthermore, inner and outer membrane fractions containing large amounts of LPS had the ability to increase phagocytosis (Table 1). This suggests that LPS can activate macrophages, as has been described previously by others (20).

There is a substantial precedence for the importance of periplasmic toxins in pathogenesis. For example, Shiga toxin of *Shigella dysenteriae* 1 (5), verotoxin (shiga-like toxin) of *E. coli* (13), and heat-labile enterotoxin of *E. coli* (7) are all periplasmic, as indicated by polymyxin release experiments. The periplasmic location of cytotoxin does not necessarily argue against a role for this toxin in the pathogenesis of *P. aeruginosa* infections. Indeed, its location in the periplasm may be part of a population strategy of *P. aeruginosa* to allow the organism to maintain its presence at an infection site. In response to either host bactericidal defense mechanisms (including complement-mediated lysis and phagocytosis) or antibiotic therapy, cytotoxin and other periplasmic constituents may be released by lysis or damage of the outer membrane. Such liberation of cytotoxin would have a significant effect on host phagocytic cells, as suggested by the previously observed killing and lysis of neutrophils (2, 25), the strong depolarization of the macrophage membrane (Fig. 3), and the reduced ability of treated macrophages to phagocytose *P. aeruginosa* (Table 1). These last two effects may indeed be analogous, since depolarization would dissipate the ion gradients that are required to signal the initiation of phagocytosis (30) and thus would result in diminished uptake, as observed from the data in Table 1. It should be noted that cytotoxin appears to be marginally more active against neutrophils compared with macrophages. While concentrations as low as 6 μg/ml have been reported to cause the complete lysis of granulocytes (3), 13 μg/ml produced com-

plete inhibition of phagocytosis but little lysis in macrophages. It is possible that macrophages display an increased resistance to this toxin.

The liberation of cytotoxin by dead and dying *P. aeruginosa* cells may be an important factor in the persistent lung infections of individuals with cystic fibrosis. Through inhibition of phagocytosis, these organisms may be capable of protecting the bacterial population from a primary defense mechanism of the lung. We are currently investigating this possibility.

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