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Interaction of Macrophage Cationic Proteins with the Outer Membrane of *Pseudomonas aeruginosa*

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The interaction of the polycationic rabbit alveolar macrophage cationic proteins MCP-1 and MCP-2 (or their identical neutrophil equivalents NP-1 and NP-2) with the surface of *Pseudomonas aeruginosa* was investigated. Both proteins bound avidly to purified lipopolysaccharide, as judged by their ability to competitively displace the probe dansyl polymyxin with 50% inhibition (I_{50}) values of 2 to 3 μM. Similar I_{50} were measured with dansyl polymyxin as a probe for cell surface binding, suggesting that the initial binding site for MCP-1 and MCP-2 on the surface of cells was lipopolysaccharide. Both MCP-1 and MCP-2 permeabilized outer membranes to the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN). The initial rate of NPN uptake plotted against the concentration of MCP-1 or MCP-2 gave sigmoidal curves, suggesting cooperative permeabilization of the outer membrane. Replotting the data as a Hill plot gave an affinity parameter, $S_{0.5}$, the concentration of MCP giving a half-maximal increase in the rate of NPN uptake, of 5 and 25 μM for MCP-1 and MCP-2, respectively, and thus subsequent studies concentrated on the more active permeabilizer MCP-1. Permeabilization of outer membranes to NPN was a function of buffer pH, with lower pH considerably favoring the permeabilizing effects of MCP-1. Thin-section electron microscopic visualization of MCP-1-treated cells showed production of extended blebs. Further evidence of an altered cell surface after MCP-1 treatment was obtained by demonstrating that treated unopsonized cells were more efficiently phagocytosed by unelicited rabbit alveolar macrophages. The data overall suggest that macrophage cationic proteins interact with the *P. aeruginosa* outer membrane in a manner typical of other polycations and suggest that one of their major functions may be to permeabilize the outer membrane.

Although phagocytosis by macrophages constitutes a major mechanism by which nonspecific resistance to infection by aspirated bacteria is achieved (7), our understanding of the biochemical mechanisms of bacterial killing is still far from complete (18). Mechanisms of macrophage antimicrobial killing fall into two distinct categories, oxygen dependent and oxygen independent. Oxygen-dependent bactericidal mechanisms include production during the respiratory burst of highly toxic oxygen derivatives such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (18). Oxygen-independent mechanisms constitute a more diverse group of antimicrobial functions, including lysosome acidification, iron-binding proteins, production of arginase, synthesis of certain components of complement, lysosomal hydrolases, including lysozyme (see reference 18 for a review), and macrophage cationic proteins (MCPs) (12).

In recent years, several cationic proteins with antibacterial activity have been identified from polymorphonuclear leukocytes (3, 4, 11, 15, 19, 20, 24, 25, 27, 28, 32–34). MCPs isolated from rabbit alveolar macrophages were first reported in 1980 (20). These peptides, named MCP-1 and MCP-2, have been sequenced. They are each 33 amino acids in length and rich in arginine and cysteine residues and differ by only a single substitution (23). Under conditions of relatively low ionic strength and at near-neutral pH, MCPs have been demonstrated to exhibit powerful antimicrobial effects against various fungal, gram-positive, and gram-negative organisms, including *Pseudomonas aeruginosa* (12, 20, 21).

P. aeruginosa is known to demonstrate high intrinsic resistance to many antibacterial agents, due in part to an inefficient hydrophilic (porin-mediated) uptake pathway across its outer membrane (8). In contrast, polycationic agents like aminoglycosides and polymyxins appear to be relatively effective at killing *P. aeruginosa*. Investigation of the mechanism of polycation interaction with the outer membrane of *P. aeruginosa* led Hancock et al. (8, 9) to propose the self-promoted uptake model. That is, polycations, such as polymyxins and aminoglycosides, interact with the outer membrane of *P. aeruginosa* by displacing the divalent cations which serve to cross-bridge adjacent lipopolysaccharide (LPS) molecules. Displacement by large polycations results in disruption of the outer membrane and consequently increased permeability to agents like lysozyme, the hydrophobic fluorophor 1-*N*-phenylnaphthylamine (NPN), and the β-lactam nitrocefin (9, 10, 13). It has been proposed that as a consequence of this perturbation of outer membrane permeability, the uptake of the polycation itself is promoted (9, 10).

Since MCPs have eight to nine arginine residues, they will be polycationic at pHs below 10. Therefore, we wished to determine whether they behaved like typical polycations in their interactions with the *P. aeruginosa* outer membrane. In this paper we present data which strongly support this concept and further suggest that at mildly acidic pHs, MCPs are functional as outer membrane permeabilizers, despite their known (12) inability to kill *P. aeruginosa* at these pHs.

MATERIALS AND METHODS

Peptide purification. MCPs were purified by a modification of the procedures of Selsted et al. (25). Phagocytic lysosomes were isolated from 10^8 elicited rabbit alveolar macrophages and extracted with fresh 10% acetic acid (25). The dialyzed 27,000 × *g* supernatant was concentrated by lyophilization and is referred to as the crude extract. The crude

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extract (10 mg/ml in 1% acetic acid) was applied to a column (2.5 by 30 cm) of BioGel P-10 (50-100 mesh; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1% acetic acid. Peptides were eluted at a flow rate of 5 ml/h, and 1-ml fractions were collected, monitored by absorbance at 214 and 280 nm, and examined on acid-urea gels (25). Fractions containing the low-molecular-weight basic peptides (i.e., MCPs) were pooled and lyophilized. Final purification and separation of MCP-1 and MCP-2 was achieved with a reverse-phase fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden) equipped with a Pro-RPC 5/10 column. Shallow acetonitrile gradients containing 0.1% trifluoroacetic acid were used in elution, and fractions were monitored at 214 nm. MCP-1 and MCP-2 eluted as discrete peaks at 13.96 and 13.98% (vol/vol) acetonitrile, respectively. In some experiments, for convenience, the source of MCP-1 and MCP-2 was 10^9 rabbit peritoneal granulocytes, which contain peptides termed NP-1 and NP-2, respectively, which are identical to the above two macrophage peptides (25). In this case, an identical purification procedure was used, although the three other characterized neutrophil basic peptides (NP-3, NP-4, and NP-5) were only separated from MCP-1 and MCP-2 on the final column, where they eluted together at 13.94% acetonitrile. Final yields and peptide concentrations were assessed by the Bio-Rad protein assay with standard I. The bactericidal activity of the peptides, as described by Lehrer et al. (12), was confirmed for the peptides purified by this method, which in our hands permitted faster purification.

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 strain H103 (2) was used in all experiments. All cultures were grown in 1% (wt/vol) proteose peptone 2 medium (Difco Laboratories, Detroit, Mich.). Experimental cultures were started for an overnight broth culture, diluted 1:50 into fresh medium, and grown at 37°C with vigorous shaking to an optical density at 600 nm of 0.4 to 0.6.

LPS isolation. LPS was isolated from strain H103 as described by Darveau and Hancock (2). The isolated LPS was extracted twice with an equal volume of chloroform-methanol (2:1) to remove trace amounts of sodium dodecyl sulfate and phospholipids which remained during the isolation procedure (2). Residual chloroform was removed by purging with nitrogen gas in a fume hood for about 30 min. A 10-mg/ml stock suspension of LPS was prepared on a dry-weight basis.

Dansyl-polymyxin binding inhibition experiments. Inhibition of dansyl-polymyxin binding to LPS was performed as described previously (18). Briefly, inhibitors of dansyl-polymyxin binding, including MCPs, were titrated into a cuvette containing 3 μg of LPS per ml and 2.5 μM dansyl-polymyxin (resulting in 85 to 90% saturation of LPS-binding sites by the dansyl-polymyxin) in 1 ml of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.2, and the decrease in the observed fluorescence (percent inhibition) was recorded. Maximum inhibition by a given compound was calculated as the extrapolated *y* axis intercept of a plot of the reciprocals of percent inhibition versus inhibitor concentration. The *x* axis intercept gave $-1/I_{50}$, where I_{50} was the concentration of inhibitor giving 50% maximal inhibition of dansyl-polymyxin binding at the LPS and dansyl-polymyxin concentrations used.

For inhibition of dansyl-polymyxin binding to whole cells (17), inhibitors were titrated into a cuvette containing 990 μl of 5 mM HEPES buffer and 10 mM sodium azide, pH 7.2; 10 μl of cells were suspended in the same buffer to an optical density at 600 nm of 0.5 with 1.5 μM dansyl-polymyxin (giving 85 to 90% saturation of cell binding sites). Maximal inhibition and I_{50} values were read from a graph of the inhibition curves.

Permeabilization of whole cells to NPN. NPN uptake assays were performed as previously described (13). Briefly, cells were prepared by washing twice in 5 mM HEPES buffer, pH 7.2, containing 1 mM KCN (to inhibit respiration and prevent active excretion of NPN [13]) and suspended to an optical density at 600 nm of 0.5 in the same buffer. The cell suspension was then allowed to sit at room temperature for 30 to 60 min before use. NPN (Sigma) was dissolved in acetone at a concentration of 500 μM and added to 1 ml of cell suspension to a final concentration of 10 μM. MCPs and crude macrophage extract were tested for their ability to permeabilize cells to NPN, and the increase in NPN fluorescence intensity was monitored continuously. Excitation and emission wavelengths were set at 350 and 420 nm, respectively, with slit widths of 5 nm. To determine the effects of pH on enhancement of NPN uptake by MCPs, bacterial cells were suspended and equilibrated before use for 30 min at 23°C in 5 mM HEPES buffer plus 1 mM KCN at pH 6.0, 6.5, 7.0, 7.5, or 8.0, and NPN uptake was assayed at these pHs. The fluorescence of NPN was unaffected by the assay pH.

Enhancement of phagocytosis by MCPs. The phagocytosis visual assay was modified from that of Speert et al. (30). Unelicited rabbit alveolar macrophages were collected and washed as before and suspended to 5×10^5 cells/ml in RPMI 1640 (Gibco, Burlington, Ontario) supplemented with 44 mM sodium bicarbonate (Fisher Scientific, Vancouver, B.C.), 10% (vol/vol) fetal calf serum (Gibco), 10 mM HEPES buffer (Terochem Laboratories, Vancouver, B.C.), 0.04% (vol/vol) 2-mercaptoethanol (Bio-Rad), 2 mM L-glutamine (Sigma), 40 U of penicillin per ml, and 40 μg of streptomycin (Gibco) per ml, pH 7.2. Samples (2 ml) of the cell suspension were incubated in Nunclon tissue culture dishes (35 by 10 mm) at 37°C in 10% CO_2 overnight. Just prior to the assay, the macrophage monolayer was gently washed twice with phagocytosis assay medium (RPMI 1640 medium supplemented with 10 mM HEPES buffer only), and then 1 ml of this medium was placed over the monolayer. Bacteria were washed and resuspended to 10^8 cells/ml. Equal concentrations of MCP-1 and MCP-2 were added to the bacteria to a final peptide concentration of 50 μg/ml, or an equal volume of sterile water was added (control). The bacteria were then added to the macrophage monolayer at a ratio of 20:1 (bacteria-macrophage), and the system was incubated for 90 min at 37°C in 10% CO_2, at which time the macrophages were scraped from the dish with a rubber policeman and gently resuspended. Slides were prepared by cytocentrifugation of 100-μl portions, followed by staining. To assess phagocytosis, the number of bacteria in each of 120 macrophages was recorded by visual inspection.

Electron microscopy. Log-phase washed (5 mM HEPES, pH 7.2) and concentrated cells of strain H103 (approximately 5×10^9/ml) were incubated with either MCP-1 (2.5 mg/ml) or crude extract (0.25 mg/ml) for 0, 10, 30, and 60 min at 5°C. A pH of 7.2 was chosen for these experiments to allow direct comparison with previous studies on polycation interaction with the outer membrane (14). Cells were then fixed in 5% glutaraldehyde and 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in Epon 812. Thin sections were cut, collected on Formvar- and carbon-coated copper grids, and stained with 1% uranyl acetate and Reynolds lead citrate. All samples were examined with a Zeiss EM10C electron microscope operating at 80 kV.

TABLE 1. Displacement, by MCPs and other cations, of dansyl-polymyxin bound to LPS and whole cells[a]

Inhibitor	Purified LPS		Whole cells	
	I_{50} (μM)	Maximal displacement (%)	I_{50} (μM)	Maximal displacement (%)
MCP-1	2.3	63	2.8	50
MCP-2	2.9	43	2.5	30
Gentamicin	72	43	36	50
Mg^{2+}	1,840	46	2,400	75

[a] I_{50}, Concentration of inhibitor resulting in competitive displacement of 50% of the maximal amount of dansyl-polymyxin displaced; maximal displacement, percentage of the total bound dansyl-polymyxin which could be displaced.

RESULTS

Dansyl-polymyxin binding to purified LPS and intact cells. Moore et al. (16) previously demonstrated the utility of dansyl-polymyxin as a probe for the cation-binding sites of LPS. Binding was accompanied by a dramatic increase in the fluorescence of the dansylated probe and at a given LPS concentration was kinetically related to the concentration of dansyl-polymyxin added until all binding sites on the LPS became saturated. The ability of other polycations and Mg^{2+} to bind to the dansyl-polymyxin-binding sites of LPS could be demonstrated by competitive displacement experiments (16), since after dissociation from LPS, dansyl-polymyxin became very weakly fluorescent.

Competitive displacement experiments were performed with MCP-1 and MCP-2 as the competing polycations (Table 1). It was shown that both MCPs had high affinities for dansyl-polymyxin-binding sites of LPS (as indicated by the I_{50} values given in Table 1) but that they bound to only a subset of the total dansyl-polymyxin-binding sites (measured as 4.3 sites per molecule of LPS in titration experiments). As controls, we confirmed our earlier observations (27) that gentamicin and Mg^{2+} also displaced dansyl-polymyxin from a portion of its LPS-binding sites (Table 1) but that polymyxin could displaced 100% of the LPS-bound dansyl-polymyxin (data not shown). Both MCPs had far greater affinities for LPS than gentamicin and Mg^{2+}.

Similar competitive displacement experiments showed that dansyl-polymyxin bound to intact cells (presumably to the divalent-cation-binding sites on LPS [17]) and could be partly displaced by MCP-1 and MCP-2. It should be pointed out that the presence of high concentrations of divalent cations at the cell surface and the association of LPS with, for example, outer membrane proteins made interpretation of these results considerably more difficult than those obtained with purified LPS. Nevertheless, we observed similar I_{50} and maximal displacement values for cells and LPS with MCP-1 and MCP-2 as well as with gentamicin and Mg^{2+}. All of the above competitive displacement experiments were repeated with LPS and whole cells from another *P. aeruginosa* strain (H215) with similar results.

Permeabilization of cells to NPN. The hydrophobic fluorescent probe NPN fluoresces weakly in an aqueous environment but strongly in a hydrophobic environment such as a membrane interior (24). NPN provides a sensitive probe for outer membrane barrier function because it is excluded from wild-type *P. aeruginosa* cells unless the outer membrane is permeabilized by a polycation (9, 13) or divalent cation chelator (9, 10), in which case NPN is taken up by cells in a time- and permeabilizer concentration-dependent fashion until the cell membranes become saturated with NPN (13).

Addition of NPN to strain H103 resulted in only a small increase in fluorescence, in confirmation of previous results (9, 13). Addition of either MCP-1 or MCP-2 resulted in a rise in NPN fluorescence until membranes became saturated (Fig. 1). At a given concentration of peptide, MCP-1 demonstrated more rapid permeabilization of cells to NPN than did MCP-2. When different concentrations of MCP-1 and MCP-2 were used in these experiments, NPN fluorescence uptake curves changed in shape from sigmoidal (like Fig. 1, curve B) to hyperbolic (e.g., Fig. 1, curve A). Although these curves were complex, the initial rates of fluorescence increase could be fitted to a Hill plot (not shown), as demonstrated previously for gentamicin promotion of NPN uptake (13). The slope of the Hill plots was greater than 1, suggesting that MCPs permeabilized strain H103 to NPN in a cooperative manner. In addition, the Hill plots yielded an affinity constant ($S_{0.5}$, the concentration of MCP at half-maximal rate of increase of NPN uptake). At pH 7.0, for MCP-1, an $S_{0.5}$ value of 5.0 μM was calculated, whereas MCP-2-permeabilized strain H103 had an $S_{0.5}$ value of 24.6 μM. Subsequent studies concentrated on the more active permeabilizer MCP-1.

Phagolysosomal pH can vary from 7.5 to 6.0 or less, depending on time after fusion of phagocytic vesicles with the lysosomes. Thus, it was of interest to determine the influence of pH on the ability of MCPs to permeabilize cells to NPN. There was a profound effect of pH on the ability of MCP-1 to permeabilize cells to NPN (Fig. 2). For example, 5 μg of MCP-1 per ml caused rapid uptake of NPN at pH 6.0 and 6.5 but extremely slow uptake of NPN at pH 7 or above. This was reflected in the calculated $S_{0.5}$ values from Hill plots, which increased nearly ninefold between pH 6 and 7.5 (Table 2). A similar effect of pH between 6 and 7 was seen for gentamicin-mediated enhancement of NPN uptake (Table 2). It must be noted, however, that these experiments did not allow us to conclude that the mechanism of MCP permeabilization involved the same sites at different pHs, although one plausible role of pH would be in deprotonation of LPS phosphates, which are thought to be the major site of polycation binding (16).

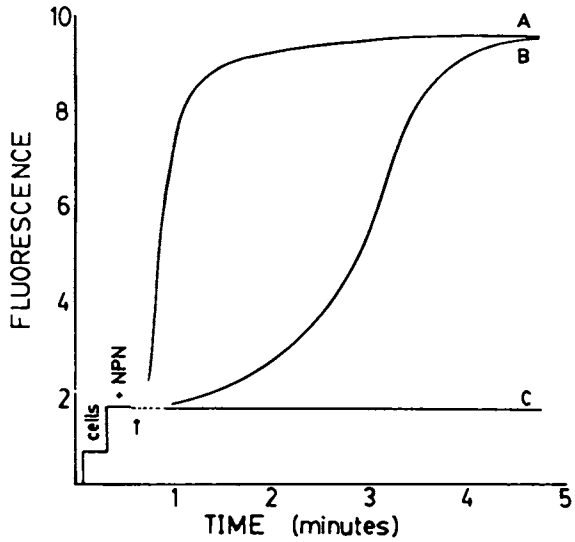

FIG. 1. Effect of MCP-1 and MCP-2 on the uptake of NPN (measured as fluorescence intensity in arbitrary units). At the arrow, 30 μg of MCP-1 (curve A) or 30 μg of MCP-2 (curve B) per ml was added. Curve C shows that in the absence of MCP-1 or MCP-2, no increase in fluorescence was observed.

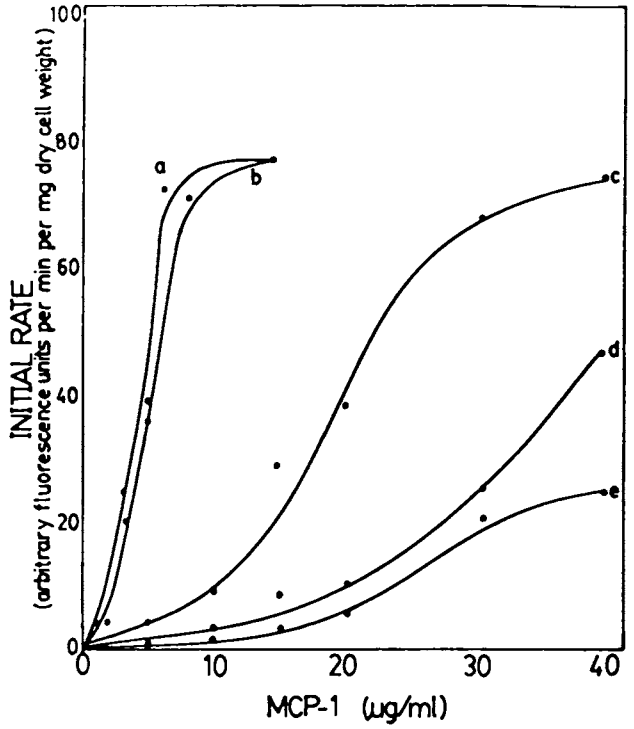

FIG. 2. MCP-1-promoted enhancement of NPN fluorescence in intact *P. aeruginosa* H103 at various pHs. Cells were washed and suspended in 5 mM HEPES buffer–1 mM KCN, pH 7.2, as shown. The assay was then performed exactly as before. Curve a, pH 6.0; curve b, pH 6.5; curve c, pH 7.0; curve d, pH 7.5; curve e, pH 8.0.

Morphological alterations of MCP-treated cells. An examination of MCP-1-treated cells by electron microscopy showed that within 5 min of initial exposure to MCP-1, the cells formed blebs of envelope material that were not observed in untreated control cells. Initially after MCP-1 addition the cell surface became very convoluted, and by 60 min numerous large outer membrane blebs formed at the poles (Fig. 3A) and along the length (Fig. 3C) of the cells. Few lysed cells were seen, indicating a lack of involvement of the inner membrane in the blebbing process. Cells treated with crude extract demonstrated blebbing similar to that of MCP-1-treated cells (data not shown).

Effect of MCP-1 on phagocytosis. Speert et al. (30) demonstrated that the extent of opsonin-independent phagocytosis of *P. aeruginosa* strains is dependent on the surface characteristics of the given strain. Since MCPs bound to the cell surface LPS and altered the barrier properties and morphology of the outer membrane, it was of interest to observe whether they could enhance phagocytosis of strain H103 in the absence of antibodies or serum. MCP-1 was chosen for these experiments because of its more dramatic effects on outer membrane properties. Addition of MCP-1 to *P. aeruginosa* H103 prior to addition to macrophages resulted in a doubling of the extent of phagocytosis compared with that of untreated strain H103 (5.59 ± 2.84 versus 2.59 ± 1.62 bacteria phagocytosed per macrophage, respectively [means ± standard deviation]; $P < 0.002$, Student's two-tailed t test). Similar highly significant ($P < 0.005$) increases in nonopsonic phagocytosis were observed after MCP-1 treatment of two other strains, H215 and H234 (data not shown).

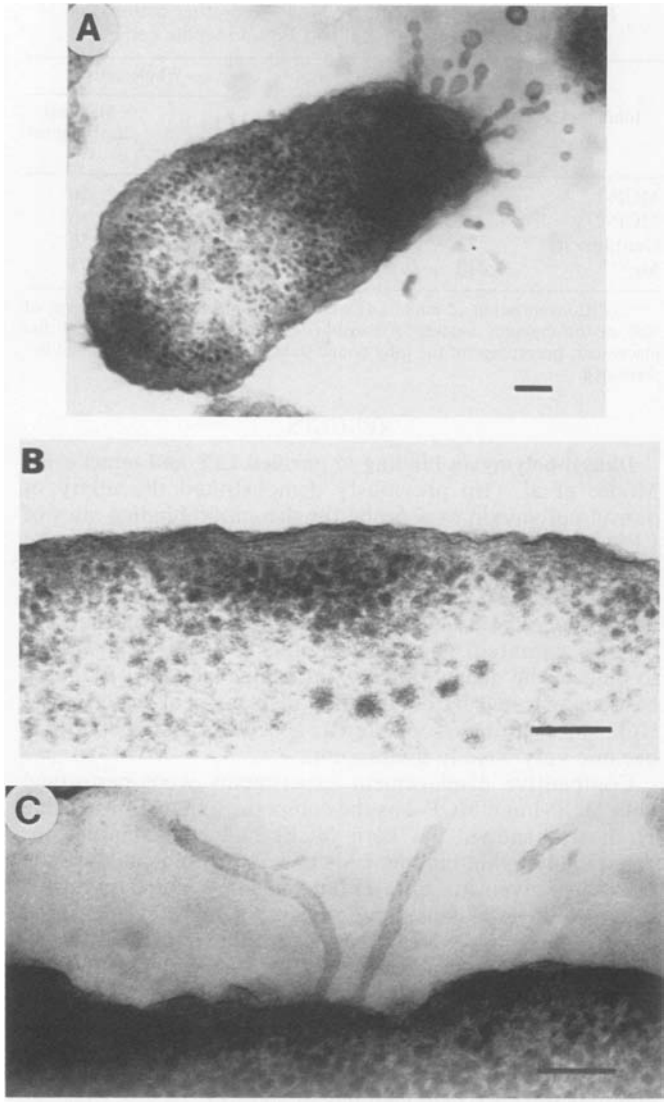

FIG. 3. Thin sections of *P. aeruginosa* showing membrane blebbing after 60 min of exposure to 2.5 mg of MCP-1 per ml (A) Whole-cell cross-section with blebs forming at the pole of the cell (the blebs run in and out of the plane of the section, resulting in the string of beads appearance); (B) high-magnification cross-section through control cell envelope; (C) high-magnification cross-section through treated cell envelope, showing blebs extending outwards from cell surface. Bars, 100 nm.

TABLE 2. Kinetics of interaction of MCP-1 and gentamicin with whole cells of *P. aeruginosa* PAO1 strain H103 to permeabilize them to NPN[a]

Permeabilizer	pH	$S_{0.5}$ (μM)
MCP-1	6.0	1.0
	6.5	1.3
	7.0	5.0
	7.5	8.8
	8.0	>10
Gentamicin	6.0	4.6
	7.0	20
	8.0	20

[a] The $S_{0.5}$ values are taken from Hill plots of the initial rate of NPN uptake (assessed as fluorescence increase) as a function of permeabilizer concentration.

DISCUSSION

Polycationic antibiotics such as polymyxins and aminoglycosides have been shown to cause an increase in the outer membrane permeability of *P. aeruginosa* (9, 10, 13). The uptake of these polycations has been hypothesized to occur via the self-promoted uptake pathway, in which polycations displace divalent cations from sites where they noncovalently cross-bridge adjacent LPS molecules (8, 9). Once the integrity of the outer membrane is disrupted, cells become permeable to agents like lysozyme and NPN (9, 13).

In accord with this theory, it was found that MCPs, being strongly polycationic, enhanced the uptake of the hydrophobic probe NPN (Fig. 1) in a manner typical of other known permeabilizers (13). Furthermore, MCPs were shown to interact with high affinity with the LPS of *P. aeruginosa* (the first requirement of the self-promoted uptake model), as demonstrated by competitive displacement of dansyl-polymyxin bound to purified LPS or whole cells (Table 1).

The role of hydrophobic interactions between other, higher-molecular-weight phagocyte bactericidal proteins and the surface of *Escherichia coli* and *Salmonella typhimurium* has been suggested (26, 31, 34). Other studies have investigated the role of LPS, finding that susceptibility of gram-negative bacteria to various bactericidal proteins increased in inverse proportion to O-antigen chain length and that deep rough mutants are more susceptible than their isogenic smooth strain parents (21, 28, 29, 31, 34).

The most interesting observation of this study was that the permeabilizing effects of MCP-1 were greatly enhanced at lower pH (Fig. 2). This was intriguing, as purified MCPs were previously shown to exhibit maximal killing of *P. aeruginosa* between pH 7.0 and 8.0, with little or no killing occurring at or below pH 6.5 (12). Although this may appear contradictory, it may define the role of MCPs during the acidification of the lysosome. After bacterial uptake by macrophages and fusion of the phagocytic vacuole to a lysosome, there is a brief transient rise in pH (1, 22), during which MCPs can kill ingested bacteria. We propose that as acidification proceeds, MCPs could permeabilize the bacterial cells, making them more susceptible to other macrophage killing systems or to degradative enzymes.

It was clear from the NPN fluorescence experiments that the surface of the bacterial cell was being modified to allow uptake of this hydrophobic probe. Furthermore, electron microscopic investigations (Fig. 3) revealed cell surface-related changes in MCP-treated cells compared with untreated cells. After exposure to MCP-1, the outer membrane was destabilized, producing numerous large blebs. Similar outer membrane blebbing, albeit with smaller blebs, has been observed in *P. aeruginosa* in the presence of other outer membrane-perturbing agents, such as EDTA (7) and the polycations polymyxin (6) and gentamicin (14), which appear to interact with divalent-cation-binding sites on LPS (8, 10). In addition, enhancement of phagocytosis of MCP-coincubated cells by rabbit alveolar macrophages also suggested that surface changes had occurred (see above). It is possible that these changes could have been the result of increased hydrophobicity due to MCP interaction (30). To investigate this possibility, partitioning of MCP-treated and untreated cells in a biphasic system of polyethylene glycol and dextran was attempted. However, no significant changes in partitioning behavior were seen (data not shown). The most plausible explanation for this was that due to the negatively charged nature of the dextran phase, any effects of an increase in surface hydrophobicity were negated by ionic interactions with dextran. However, the fact that little or none of the hydrophobic probe NPN was taken up by whole cells before MCP addition is in agreement with the concept of increased surface hydrophobicity due to MCP interaction with the bacterial cell surface. This then would explain the enhancement of phagocytosis by MCP-1, but we cannot rule out the possibility that MCP-1 acted as an opsonin. Nevertheless, these results may be physiologically significant, since the equivalent peptides, defensins, can be secreted from human neutrophils after activation (7).

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