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The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*

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Summary

A cecropin/melittin hybrid peptide (CEME) produced by recombinant DNA procedures was tested for its ability to interact with the outer membrane of *Pseudomonas aeruginosa* and found to have identical biological properties to that of chemically synthesized CEME. CEME was shown to kill *P. aeruginosa* and permeabilize its outer membrane to lysozyme and 1-*N*-phenylnaphthlyamine, in some cases better than other antimicrobial agents and permeabilizers. CEME demonstrated a high-binding affinity to purified *P. aeruginosa* lipopolysaccharide (LPS) and LPS in whole-cell environments. These data provide information on the molecular mechanism of CEME antimicrobial activity and strongly suggest that it is taken up across the outer membrane by the self-promoted uptake pathway.

Introduction

The resistance of *Pseudomonas aeruginosa* to many conventional antibiotics can be attributed in part to its low outer membrane permeability (Nikaido and Vaara, 1985; Hancock and Bell, 1989). Small hydrophilic substances can pass with low efficiency through the water-filled channels of a class of proteins called porins. The effectiveness against *P. aeruginosa* of certain polycationic antibiotics such as polymyxin B and gentamicin can be explained by their ability to access another uptake mechanism, termed the self-promoted uptake pathway (Nicas and Hancock, 1980; Hancock *et al.*, 1981). This uptake mechanism is initiated when the polycation binds to divalent cation binding sites of lipopolysaccharide (LPS) molecules of the outer membrane, competitively displacing the divalent cations that stabilize adjacent LPS molecules. This results in outer membrane destabilization and the subsequent uptake of the interacting polycation. This hypothesis is supported by the fact that polymyxin B has a high affinity for LPS (Moore *et al.*, 1986) and is able to permeabilize outer membranes to probe molecules such as the protein lysozyme and the fluorescent probe 1-*N*-phenylnaphthylamine (NPN) (Hancock and Wong, 1984).

Over the past decade, a new class of polycationic antibiotics has been discovered and examined. It consists of small antimicrobial peptides that are found in many different species of mammals and insects. This class can be further divided into two groups based on structural features. The first group consists mainly of β-sheet peptides that contain one or more disulphide bonds. Included in this group are the mammalian and insect defensins (Hoffmann and Hetru, 1990; Lehrer *et al.*, 1993) and charybdotoxin (Miller *et al.*, 1985). The other main group of peptides exist as random-coiled structures in aqueous solutions but adopt an α-helical structure upon interaction with membranes. Examples of these peptides are cecropins (Steiner *et al.*, 1981), magainins (Zasloff, 1987) and melittin (Habermann and Jentsch, 1967). In addition to these naturally occurring peptides, many analogues and hybrids have been synthesized in an attempt to improve antimicrobial activity (Andreu *et al.*, 1992; Wade *et al.*, 1992). All these peptides show a broad range of antimicrobial activity (Wade *et al.*, 1992; Lehrer *et al.*, 1993) and have been shown to form voltage-dependent ion-permeable channels in planar lipid membranes (e.g. Christensen *et al.*, 1988). Sawyer *et al.* (1988) were the first to examine the interaction of defensins (rabbit macrophage cationic peptides) with the outer membrane of *P. aeruginosa*. They demonstrated that MCP-1 and MCP-2 had high binding affinities for purified LPS and whole cells. In addition the two peptides were able to permeabilize the *P. aeruginosa* outer membrane to NPN, consistent with the subsequent studies of Lehrer *et al.* (1989) who showed that human neutrophil peptide 1 permeabilized the *Escherichia coli* outer membrane. These data led to the proposal that defensins were taken up by the self-promoted uptake pathway (Sawyer *et al.*, 1988). There have been, however, only limited studies on the interaction

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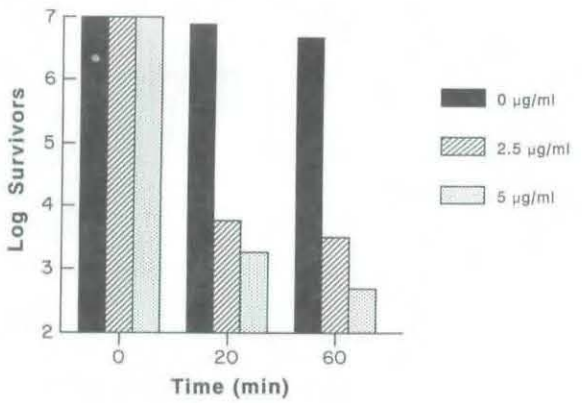

Fig. 1. *In vitro* killing of *P. aeruginosa* K799 by recombinant CEME. *P. aeruginosa* K799 (10^7 c.f.u. ml^{-1}) was incubated with 0, 2.5, or 5.0 μg ml^{-1} of recombinant CEME for 0, 20 or 60 min before plating the bacteria on LBNS plates to obtain viability counts. The data presented are the average of two trials with approximately 10% variation between experiments.

of α-helical peptides with the outer membrane, in spite of their potent antibacterial activity against Gram-negative bacteria.

Recently, we described the bacterial production of a recombinant cecropin/melittin hybrid peptide (Piers *et al.*, 1993), hereafter referred to as CEME. This recombinant peptide has the same amino acid content, *N*-terminal amino acid sequence, and gel electrophoretic mobility as CEME produced by peptide synthesis methods. In this study, we investigated the mechanism of interaction of CEME with the Gram-negative bacterium *P. aeruginosa*.

Results

Killing of P. aeruginosa *K799 by CEME*

The antimicrobial activity of recombinant CEME was initially quantified using an *in vitro* killing assay (Lehrer *et al.*, 1983) and the data were plotted as the log c.f.u. ml^{-1} surviving as a function of time (Fig. 1). The results showed that *P. aeruginosa* K799 was highly susceptible to the action of CEME, with greater than 99.9% of the bacteria killed by 2.5 μg ml^{-1} of peptide in 20 min. Under these conditions, CEME was able to kill *P. aeruginosa* more effectively (a decrease in viability of 3.2 log orders) than the reported value for rabbit lung macrophage cationic protein 1 (2.1 log order decrease; Lehrer *et al.*, 1983). Further incubation caused little or no decrease in viability compared to the control. This could be due to interference by bacterial components, such as LPS, released from initially lysed cells, or it may represent a phenotypically less susceptible subpopulation of cells.

Minimum inhibitory concentration studies

Recombinant CEME was tested for its ability to inhibit the growth of various *P. aeruginosa* strains compared to polymyxin B (a cyclic cationic peptide with a hydrophobic tail), gentamicin (an aminoglycoside) and ceftazidime (a β-lactam). The results (Table 1) showed that recombinant CEME and chemically synthesized CEME possessed identical antibacterial activities against these organisms. When the minimal inhibitory concentrations (MICs) against *P. aeruginosa* strain H309 were converted to μM (polymyxin B, 0.3 μM; gentamicin, 1.0 μM; ceftazidime, 1.8 μM; CEME, 0.9 μM) the results indicated that CEME was more effective at inhibiting the growth of this organism on a molar basis than the often utilized anti-pseudomonal antibiotics gentamicin and ceftazidime. Strain Z61 has multiple outer membrane alterations that result in an increased susceptibility to most antibiotics (Angus *et al.*, 1982). The fourfold greater susceptibility of this mutant to CEME indicated that the outer membrane of wild-type *P. aeruginosa* was a barrier to penetration of CEME.

One of the key proposals of the self-promoted uptake hypothesis is the initial interaction between the cationic antibiotic and negatively charged sites on the surface of the outer membrane (Hancock *et al.*, 1981). In cells grown under physiological conditions, these sites are occupied by divalent cations (usually Mg^{2+} or Ca^{2+}). Therefore, one can envision that, in the presence of Mg^{2+} ions, the MIC of compounds proposed to be taken

Table 1. Minimum inhibitory concentration determination for recombinant CEME against *P. aeruginosa*.

			MIC (μg ml^{-1})[a]				
Strain	Relevant phenotype	Addition	PX	GM	CEF	rCEME	sCEME
H309	Wild type	None	0.5	1	1	2.4	2.4
H309	Wild type	5 mM Mg^{2+}	1	4	2	38.4	38.4
H309	Wild type	80 mM Na^{+}	0.5	1	2	4.8	4.8
K799	Parent of Z61	None	0.5	1	2	4.8	4.8
Z61	Antibiotic supersusceptible	None	0.06	0.25	0.03	1.2	1.2

a. PX, polymyxin B; GM, gentamicin; CEF, ceftazidime; rCEME, recombinant CEME; sCEME, chemically synthesized CEME.

up by the self-promoted uptake pathway would increase owing to the Mg^{2+} ions competing for these negatively charged binding sites. Indeed it has been demonstrated that the presence of 5 mM $MgCl_2$ can increase the MIC of polymyxin B fourfold (Nicas and Hancock, 1980). The MIC determination was repeated for strain H309 in the presence of 5 mM Mg^{2+} or 80 mM Na^+ to determine whether or not the antibacterial activity of CEME would be inhibited (Table 1). The results showed that the MIC of CEME was increased 16-fold in the presence of Mg^{2+} but only minimally in the presence of a higher concentration of Na^+. Other antibiotics were also affected by the presence of Mg^{2+}, albeit to a lesser extent. This evidence was consistent with the hypothesis that the initial step in the antibacterial mechanism of CEME is an association with the negatively charged sites on the cell surface.

Synergy between CEME and other antibiotics

The ability of CEME to augment the activity of different antibiotics was tested. Previous studies have shown that at sub-MIC levels, some cationic membrane-permeabilizing agents such as PMBN, a delipidated derivative of polymyxin B, and $lysine_{20}$ were able to increase the sensitivity of bacteria to a number of different antibiotics (Vaara and Vaara, 1983). Therefore, MIC assays using various antibiotics were performed in the presence of 1/2 or 1/4 MIC levels of CEME to determine whether any synergy existed between them. CEME had no effect on the MIC of antibiotics that are proposed to be taken up through porins (ceftazidime, imipenem, and tetracycline), or believed to cross the membrane via a hydrophobic uptake pathway (novobiocin and fusidic acid). Levels of 0.6 $\mu g\,ml^{-1}$ and 1.2 $\mu g\,ml^{-1}$ CEME rendered strain H103 two- and fourfold more susceptible, respectively, to polymyxin B. These results are consistent with the synergistic effects of the membrane permeabilizing (see below) and killing activities of these two compounds.

Membrane permeabilization studies

Compounds taken up by the self-promoted uptake pathway have been demonstrated to permeabilize the outer membrane to various probe molecules (Hancock *et al.*, 1981; Loh *et al.*, 1984). The outer membrane-permeabilizing activities of many different compounds have been studied using these probes (Hancock and Wong, 1984; Vaara, 1992). One of them, lysozyme, is a 14 kDa basic protein that is unable to penetrate intact outer membranes, but can diffuse across disrupted membranes to exert its lytic activity (Hancock and Wong, 1984). Because of its large size, one would expect that significant destabilization of the membrane would be required for it to penetrate to its peptidoglycan substrate. CEME was tested for its ability to facilitate the uptake of lysozyme by permeabilizing the outer membrane of *P. aeruginosa* (Fig. 2). At low concentrations, CEME demonstrated better outer membrane permeabilizing activity than polymyxin B, but at higher concentrations, polymyxin B was a stronger permeabilizer. However, as with the MIC data, when the concentrations were converted to μM, CEME was significantly better than polymyxin B at enhancing the uptake of lysozyme across the *P. aeruginosa* outer membrane. CEME was also found to have membrane-permeabilizing activity that was 10- to 20-fold higher than gentamicin (Hancock *et al.*, 1981; data not shown). Lysis assays were also performed in the absence of lysozyme (Fig. 2, open symbols), which revealed that CEME by itself possessed slight lytic activity at higher concentrations. Additional experiments demonstrated that Mg^{2+} concentrations as low as 1 mM were able to prevent lysozyme lysis induced by CEME (data not shown), presumably by competitively inhibiting the interaction of CEME with the Mg^{2+}-binding sites of the LPS.

A second probe molecule, NPN, is an uncharged, hydrophobic fluorescent probe that has been used to study membrane permeabilization (Loh *et al.*, 1984; Sawyer *et al.*, 1988). When NPN is mixed with cells, it fluoresces weakly since it is unable to breach the outer membrane

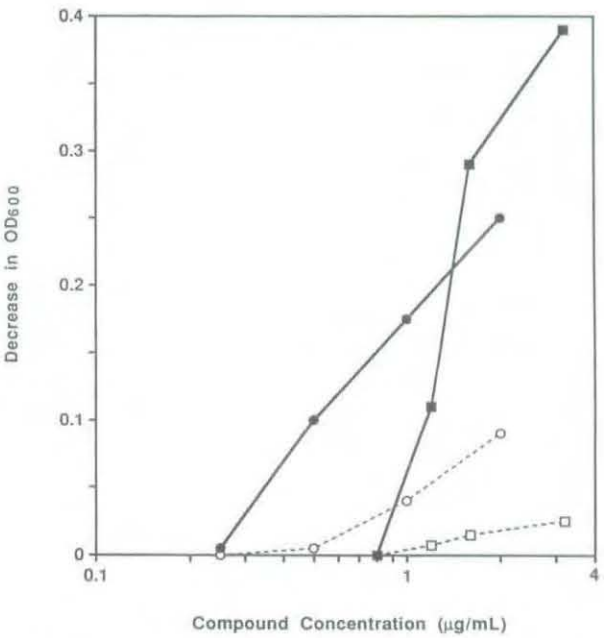

Fig. 2. Peptide-mediated lysozyme lysis of *P. aeruginosa* H309. Various concentrations of polymyxin B (squares) and CEME (circles) were tested for their ability to lyse *P. aeruginosa* H309 cells in the presence (closed symbols) or the absence (open symbols) of 50 $\mu g\,ml^{-1}$ of lysozyme. Lysis was measured as a decrease in OD_{600}. A representative experiment from three independent trials is presented.

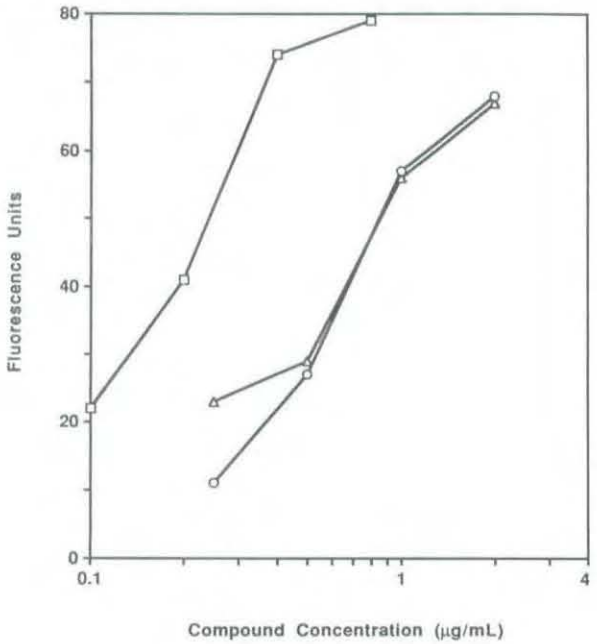

Fig. 3. Peptide-mediated NPN uptake in *P. aeruginosa* H309. *P. aeruginosa* H309 cells were incubated with NPN in the presence of various concentrations of polymyxin B (squares), recombinant CEME (circles) or chemically synthesized CEME (diamonds). Enhanced uptake of NPN was measured by an increase in fluorescence caused by the partitioning of NPN into the hydrophobic membrane. A representative experiment from three independent trials is presented.

permeability barrier. Upon membrane destabilization, however, it can partition into the hydrophobic environment of the membrane where it emits a bright fluorescence. NPN is both smaller and more hydrophobic than lysozyme, which enables it to insert into membranes more easily than lysozyme (Loh *et al.*, 1984). Therefore it may be able to detect more subtle disruptions of the outer membrane. Figure 3 shows the results of NPN-uptake assays performed on *P. aeruginosa* H309. Recombinant CEME and synthetic CEME showed virtually identical permeabilizing activities. Polymyxin B, however, was able to enhance NPN uptake into the *P. aeruginosa* outer membrane at lower concentrations than CEME, in contrast to the data observed in the lysozyme lysis assays. Similar concentrations (0.5–2.0 $\mu g\,ml^{-1}$) of CEME permeabilized the *P. aeruginosa* H309 outer membrane to lysozyme and NPN. In contrast, the lowest concentrations of polymyxin B that were able to permeabilize cells to NPN were not sufficient to permeabilize them to lysozyme.

Interaction of CEME with LPS

According to the self-promoted uptake model, compounds that access this pathway initially bind to the divalent-cation-binding sites of LPS. To study this, dansyl polymyxin B displacement assays were performed using *P. aeruginosa* H309 purified LPS or whole cells. Dansyl polymyxin B has been shown to bind to the divalent-cation-binding sites of LPS, resulting in greatly enhanced fluorescence of the dansyl group (Moore *et al.*, 1984). This property led to the development of an assay for determining the relative LPS binding affinities of antibiotics based on their ability to competitively displace dansyl polymyxin B from LPS (Moore *et al.*, 1986). Dansyl polymyxin B was added to a sample of LPS until approximately 90% of the binding sites were occupied as indicated by 90% of maximal fluorescence enhancement. CEME or other polycations were then titrated in and displacement of the dansyl polymyxin B monitored by the decrease in fluorescence. To quantify these affinities, the I_{50} value, which was the concentration of compound that resulted in 50% maximal displacement of the dansyl polymyxin B, was calculated for each compound (Table 2). The I_{50} values showed that polymyxin B and CEME had similar affinities for purified LPS. Both, however, demonstrated substantially higher affinities for LPS than did gentamicin and Mg^{2+}. To test whether these compounds bind to LPS in intact cells, the assay was repeated using *P. aeruginosa* H309 whole cells instead of purified LPS. It appeared from the calculated I_{50} values (Table 2) that CEME showed a twofold higher affinity for cellular LPS than polymyxin B. However, one must interpret such conclusions cautiously because of the complexity of the whole-cell system. The levels of competing divalent cations, the possible release of LPS molecules, the influence of dansyl polymyxin B, which itself perturbs the permeability barrier, and the effects of CEME on membrane structure are all complexities that may affect the results of such whole-cell competitive displacement experiments. Nevertheless, these data indicated that CEME was able to bind strongly to LPS in the context of whole cells.

Discussion

The study of cationic peptides such as cecropins and defensins has been somewhat limited by the paucity of

Table 2. I_{50} values (μM) for various compounds against *P. aeruginosa* LPS and whole cells.[a]

Compound	*P. aeruginosa* H103 LPS	*P. aeruginosa* H309 whole cells
Polymyxin B	0.93 ± 0.03	0.85 ± 0.13
CEME	1.30 ± 0.37	0.41 ± 0.08
Gentamicin	12.17 ± 0.58	19.0 ± 5.2
$MgCl_2\ 6H_2O$	850 ± 132	127 ± 31

a. Each value is the average of at least three trials ± the standard deviation.

available material and the complexity of their purification schemes. Although chemical synthesis has been used to make small peptides like NP-1 (Rao *et al.*, 1992) and cecropin A (Boman *et al.*, 1989), for larger peptides and those with complex disulphide arrays this is not feasible. Therefore, we devised a procedure for the production and purification of an α-helical cecropin/melittin hybrid peptide, CEME, using *Staphylococcus aureus* as the host organism and truncated protein A as a fusion partner (Piers *et al.*, 1993). CEME peptides produced by either this recombinant procedure or by chemical synthesis had identical amino acid contents, *N*-terminal amino acid sequences, and electrophoretic mobilities on acid urea gels (Piers *et al.*, 1993). In this report we have further demonstrated that these two peptides have the same antimicrobial and membrane-permeabilizing activities against *P. aeruginosa*, confirming that recombinant procedures are a viable alternative to chemical synthesis for producing these types of peptides.

Until recently, very few studies had investigated the interaction of cationic peptides with the outer membrane of Gram-negative bacteria. Sawyer *et al.* (1988) proposed that rabbit defensins, and perhaps other cationic peptides, cross the outer membrane via the self-promoted uptake pathway that was originally proposed for other cationic compounds (Hancock *et al.*, 1981). Our work provides evidence that the α-helical peptide CEME is also taken up by this pathway.

Compounds taken up by the self-promoted uptake pathway have been shown to permeabilize the outer membrane (Hancock *et al.*, 1981). With respect to cationic peptides, and defensins in particular, there has been some disagreement as to whether or not they possess the ability to disrupt membranes. Rabbit defensins (Sawyer *et al.*, 1988), human defensins (Lehrer *et al.*, 1989) and bactenicins (Skerlavaj *et al.*, 1990) were all shown to permeabilize the outer membrane. In contrast, Viljanen *et al.* (1988) argued that sub-lethal concentrations of human defensins could not permeabilize the outer membrane to rifampicin as measured by fractional inhibitory concentration (FIC) assays. The results of Figs 2 and 3 demonstrated that sub-lethal concentrations of recombinant CEME were able to permeabilize the outer membranes of *P. aeruginosa* to lysozyme and NPN. Interestingly, while CEME had permeabilizing activity that was similar for both NPN and lysozyme, the lower concentrations of polymyxin B that permeabilized outer membranes to NPN were unable to permeabilize them to lysozyme. These data could be explained if polymyxin B caused more subtle membrane perturbations at low ($<1\,\mu g\,ml^{-1}$) concentrations and larger disruptions at higher ($>1\,\mu g\,ml^{-1}$) concentrations. The similarities of CEME activity in both assays suggested that the peptide caused substantial perturbations at both low and high concentrations.

Given the ability of CEME to perturb the outer membrane at moderate concentrations, it was interesting to find that at such concentrations it was unable to augment the antimicrobial activity of antibiotics such as novobiocin and fusidic acid that are believed to traverse the outer membrane via the hydrophobic pathway. It is possible that owing to the high antimicrobial activity of CEME, concentrations that were required to permeabilize the outer membrane to such antibiotics, resulted in cell death and thus masked the permeabilizing activity. This has also been shown for polymyxin B, which could not enhance the uptake of novobiocin or fusidic acid (Vaara and Vaara, 1983), despite evidence that it could permeabilize outer membranes at moderate concentrations (this study; Hancock and Wong, 1984). Interestingly, PMBN, with an MIC of $\geqslant 100\,\mu g\,ml^{-1}$, was able to sensitize *E. coli* and *Salmonella typhimurium* to hydrophobic antibiotics, presumably because there was no bacterial killing to mask the effect (Vaara and Vaara, 1983).

Another proposal of the self-promoted uptake hypothesis is the ability of the compounds that access this pathway to bind to the divalent-cation-binding sites of LPS. This is proposed to promote a localized destabilization of the outer membrane leading to the uptake of the destabilizing molecule. Previous studies have demostrated the ability of defensins (Sawyer *et al.*, 1988), magainins (Rana *et al.*, 1990), and melittin (David *et al.*, 1992) to interact directly with LPS. This study provides evidence that CEME interacts with LPS at the negatively charged sites that are occupied by divalent cations under physiological conditions. First, the presence of Mg^{2+} in MIC assays rendered *P. aeruginosa* more resistant to the action of cationic peptides (Table 1), presumably because of competition for divalent-cation-binding sites. Second, the ability of the cationic peptides to permeabilize the outer membrane to compounds such as lysozyme was inhibited by Mg^{2+}. Third, dansyl polymyxin B that was displaced from LPS by the cationic peptides could also be displaced by Mg^{2+} (Table 2) a result consistent with a common binding site for all of these compounds.

It has been previously demonstrated that different compounds which access self-promoted uptake, e.g. polymyxin B and gentamicin, are affected differentially by mutations and by competing divalent cations, and are able to permeabilize membranes to differing extents (Nicas and Hancock, 1980; Moore *et al.*, 1986; Hancock *et al.*, 1981; Sawyer *et al.*, 1988). Such differential effects were also observed here for polymyxin and CEME in that Mg^{2+} affected the MIC of polymyxin twofold and that of CEME 16-fold whereas differences were also observed in the ability of these compounds to enhance uptake of the probes NPN and lysozyme. There are several possible causes for such heterogeneity. These include (i) intrinsic structural differences in the interacting molecules

(e.g. polymyxin B has a fatty acyl tail that might be important in promoting interaction *in vivo* and would be unaffected by divalent cation competition), (ii) the multiplicity of potential interaction sites on LPS and the heterogeneity of such sites (Moore *et al.*, 1986), and (iii) the varied complexities of the model systems and heterogeneity of the probes used in these model systems. Nevertheless we feel that despite quantitative differences, the observed qualitative similarities suggest similar mechanisms of uptake for CEME and polymyxin B. Based on all these observations, we conclude that CEME crosses the outer membrane of *P. aeruginosa* by the self-promoted uptake pathway. It subsequently causes cell death, perhaps by forming channels in the cytoplasmic membrane.

Experimental procedures

Strains, plasmids and growth conditions

P. aeruginosa H309 was a derivative of PAO1 strain H103 and contains the plasmid RP1 (Hancock, 1984). *P. aeruginosa* K799 and its isogenic antibiotic-supersusceptible mutant Z61 have been previously described (Angus *et al.*, 1982). *S. aureus* strain RN4220 (Kreiswirth *et al.*, 1983) was a gift from S. Kahn. Most strains were grown on Luria Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract) supplemented with 0.5% (w/v) NaCl (LBNS) and 1.5% (w/v) agar. For all assays and MIC determinations, bacteria were grown in LB without any salt supplement. The plasmid pPA-CEME (Piers *et al.*, 1993) was a derivative of pRIT5 (Nilsson *et al.*, 1985) containing a synthetic oligonucleotide cartridge that encodes the CEME peptide preceded by a methionine codon. This plasmid was maintained in *S. aureus* using 10 $\mu g\,ml^{-1}$ of chloramphenicol.

Reagents

Chemically synthesized CEME was obtained from the Alberta Peptide Institute (Edmonton, Alberta). Polymyxin B, gentamicin sulphate, lysozyme and NPN were purchased from Sigma.

Production and purification of CEME

CEME was isolated as previously described by Piers *et al.* (1993). Briefly, *S. aureus* RN4220(pPA-CEME) was grown in LBNS to an OD_{600} of 1.8–2.0 and the cells removed by centrifugation and filtration. The protein A/CEME fusion protein (PA/CEME) was purified from the culture supernatant using IgG-Sepharose (Pharmacia) as previously described (Uhlén *et al.*, 1984). The CEME peptide was released from PA/CEME by CNBr cleavage and partially purified by gel exclusion chromatography using Bio-Gel P100 (Bio-Rad). Final purification of the peptide was carried out on a PepRPC FPLC column (Pharmacia) using an acetonitrile gradient. The pure peptide was quantified using a protein assay that detects free amino groups (Bader and Teuber, 1973).

In vitro *killing assay*

This assay was performed as described previously (Lehrer *et al.*, 1983). Briefly, reactions were carried out in 100 µl volumes and contained 10^6 c.f.u. of *P. aeruginosa* K799 in a low-ionic-strength buffer (10 mM potassium phosphate, pH 7.4), and either 2.5 $\mu g\,ml^{-1}$ or 5.0 $\mu g\,ml^{-1}$ of CEME. After either 20 or 60 min at 37°C, samples of the bacteria were removed, diluted and plated to obtain a viable count.

Minimum inhibitory concentration

These assays were carried out according to the broth-dilution method (Amsterdam, 1991). Briefly, cells were grown overnight at 37°C in LB and diluted in the same medium to give concentrations of approximately 10^4 to 10^5 c.f.u. ml^{-1}. Serial dilutions of the antimicrobial substances in LB were set up in a 96-well microtitre plate. Subsequently, 10 µl of bacteria were pipetted into 100 µl volume of the diluted antibiotic, and the plates incubated overnight at 37°C. Samples of the bacteria were plated to ensure they were within the proper inoculum range. The next day the microtitre plates were scored for growth in the wells, and the MIC determined as the lowest antibiotic concentration that inhibited growth. To determine the effect of cations on the MIC values of the various compounds, either 5 mM $MgCl_2$ or 80 mM NaCl were included in the LB medium. In the synergy MIC studies, the given sub-MIC concentrations of CEME were included in the LB-S medium.

Lysozyme lysis assays

The uptake of lysozyme into whole cells as a result of membrane permeabilization by various compounds was previously described by Hancock *et al.* (1981). An overnight culture of *P. aeruginosa* H309 grown in LB was diluted into fresh medium and grown to an OD_{600} of 0.5–0.6. The cells were harvested, washed once with one volume of assay buffer (5 mM HEPES pH 7.2, 5 mM KCN), and resuspended in the same buffer to an OD_{600} of 0.5. Assays consisted of 600 µl of cells with 50 $\mu g\,ml^{-1}$ of chicken egg white lysozyme and varying concentrations of cationic compounds. Cell lysis was measured in a spectrophotometer as a decrease in the OD_{600}. Parallel experiments performed without lysozyme enabled the measurement of the lytic activity of the compounds themselves. To test whether or not permeability to lysozyme could be inhibited by divalent cations, various concentrations of $MgCl_2$ were added to the assay after the addition of lysozyme and before the addition of the test compound.

1-N-*phenylnaphthylamine uptake*

This assay was previously described by Loh *et al.* (1984). Cells were prepared as for the lysozyme lysis assay. The assay mixture consisted of 1 ml of cells and 10 µM NPN. Various concentrations of cationic compounds were added to the cuvette, and the increase in NPN fluorescence owing to its partitioning into the outer membrane was measured in a fluorescence spectrophotometer. Control experiments,

similar to those reported previously (Loh *et al.*, 1984), demonstrated that fluorescence enhancement was due to NPN uptake by cells. Permeabilizing activity was designated as the total fluorescence minus the fluorescence caused by NPN in the absence of cationic substances. Following the fluorescence measurement, the OD_{600} of the cells was taken, to confirm that no significant cell lysis had occurred in the presence of the peptide.

Dansyl polymyxin B displacement assays

P. aeruginosa H103 LPS was isolated by Dr Nedra Karunaratne using the method of Darveau and Hancock (1983), and dansyl polymyxin B was synthesized by Susan Farmer as previously described (Schindler and Teuber, 1975). The dansyl polymyxin B displacement assays were performed as described by Moore *et al.* (1986). Briefly, the assay mixture consisted of 1 ml of 5 mM HEPES pH 7.2 containing $3\,\mu g\,ml^{-1}$ of LPS and 2.5 μM of dansyl polymyxin B, which resulted in 90–100% maximum fluorescence as measured by a fluorescence spectrophotometer. Samples (5 μl) of the test compounds were added and the subsequent decrease in fluorescence owing to displacement of the dansyl polymyxin B from the LPS was recorded after each addition. The data were plotted as the fraction of dansyl polymyxin B bound versus the compound concentration (I). The relative affinities of the compounds for the binding sites on LPS were determined by reading the I_{50} values off the graph. I_{50} represented the concentration of compound that resulted in 50% maximal displacement of the dansyl polymyxin B from the LPS. For dansyl polymyxin B binding inhibition assays using whole cells, *P. aeruginosa* H309 cells were prepared in the same way as for the lysozyme lysis assay. This assay consisted of 10 μl of cells at an OD_{600} of 0.5, 990 μl of 5 mM HEPES pH 7.2, 5 mM KCN and a concentration of dansyl polymyxin B that had been determined to result in 90–100% binding saturation. Cationic compounds were titrated in, and I_{50} values determined as described above. All experiments were performed a minimum of three times.

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