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THE MORPHOLOGICAL EFFECTS OF TWO ANTIMICROBIAL PEPTIDES, HECATE-1 AND MELITTIN, ON *ESCHERICHIA COLI*

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Abstract

The effects of the 26 amino acid, cationic, amphipathic, antibacterial peptide melittin and hecate-1, a 23 amino acid analog of it, on the gram negative bacterium *Escherichia coli* were investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and freeze-fracture. Both peptides killed virtually all bacteria at the peptide concentration and cell density used. TEM and SEM revealed aggregates of bacteria entangled with material extruded from the bacterial surfaces. SEM revealed irregular bacterial surfaces with bleb-like projections. TEM and freeze-fracture indicate that the bacterial inner and outer membranes, as well as the peptidoglycan layer between, were extensively damaged. The cytoplasmic contents of the cells, however, did not appear radically disturbed, providing little evidence for osmotically induced cytolysis.

Key words: Melittin, hecate, antibacterial peptide, cytolysis, lytic peptide, *Escherichia coli*, antimicrobial.

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Introduction

The membranes of many cells are known to be profoundly disordered by a family of small, cationic, amphipathic peptides with the capacity to form α-helical structures (see Maloy and Kari, 1995 for a general review of defensive peptides). These peptides include antimicrobial peptides produced by insects (e.g., cecropin, reviewed by Boman, 1991), amphibians (e.g., magainin, reviewed by Zasloff, 1992), and mammals (e.g., cecropin-P, Lee *et al.*, 1989).

Melittin, the principle toxic component of honeybee (*Apis mellifera*) venom is also such an anti-microbial peptide (Boman *et al.*, 1989). Melittin is a 26 amino acid peptide with a positively charged amphipathic C-terminus, a hydrophobic N-terminus, and powerful cytolytic activity against eukaryotic and prokaryotic cells (Katsu *et al.*, 1989; Dempsey, 1990). Several mechanisms designed to account for its cytotoxic and anti-microbial effects have been proposed but none is universally accepted (Dempsey, 1990; Weaver *et al.*, 1992). Prominent mechanisms advanced include its activity in the formation of pores or channels in membranes followed by ionic imbalance and osmotic cytolysis (Tosteson *et al.*, 1989, 1990). Pores or channels are a likely result of peptide insertion into the membrane and subsequent formation of transmembrane peptide-peptide aggregates in which the hydrophobic face of each helix interacts with the hydrophobic constituents of the membrane, while each hydrophilic surface faces inward to form an ion-channel or pore (Tosteson *et al.*, 1989, 1990). A second group of mechanisms proposes wedge-shaped partial insertion of monomers into the membrane, followed either by osmotic cytolysis (Dawson *et al.*, 1978) or by non-osmotic membranolysis resulting from the induction of a catastrophic curvature in the membrane (Weaver *et al.*, 1992). Other proposed mechanisms involve the aggregation of membrane proteins (Hui *et al.*, 1990), the expulsion of phospholipids (Katsu *et al.*, 1989), and membrane micellization (Dufourcq *et al.*, 1986).

Although some investigations of melittin-membrane

interactions have included limited morphological observations on peptide treated erythrocytes (Dempsey, 1990; Hui *et al.*, 1990) or on multilamellar phospholipid vesicles (Dufourcq *et al.*, 1986), none contains direct observations of its interactions with bacteria. In the present study, we investigated the morphological effects of melittin and a 23 amino acid synthetic melittin analog (hecate-1) on the gram negative bacterium *Escherichia coli*.

Materials and Methods

Peptides

Purified melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) with less than 5 units phospholipase A_2 per mg was obtained from Sigma (St. Louis, MO) and further purified using reverse phase HPLC as previously described (DeGrado *et al.*, 1981). Hecate-1 (FALALKALKKALKKLKKALKKAL) was synthesized on a MilliGen 9050 (Marlborough, MA) peptide synthesizer using Fmoc chemistry, purified using reverse phase HPLC, and its purity confirmed using mass spectrometry. The percent peptide mass used in calculating concentrations was obtained from quantitative amino acid analysis of the HPLC product.

Bacterial culture, peptide treatment and fixation

Escherichia coli (NM554) were routinely cultured overnight in brain heart infusion broth to a reproducible cell density based on optical density and confirmed by plate counting. The bacteria were washed in 150 mM sodium chloride to remove medium, especially divalent cations. Treatment with peptide lasted 15 minutes at room temperature and was accomplished by adding 100 μl of washed stock bacteria added to 100 μl of 1 mM peptide in 800 μl of cation-free diluent (150 mM NaCl in HEPES buffer at pH 7.4) to produce a final concentration of 100 μM peptide and 10^8 bacteria in 1 ml. Maintaining approximately the same number of bacteria as well as the same concentration of peptide in all experiments is essential to obtaining reproducible results. The 100 μM peptide concentration was chosen because virtually 100% of the 10^8 bacteria are killed at this concentration based on post-treatment plate counts. Lower concentrations (e.g., 50 μM) peptide resulted in incomplete killing at this cell density. Lower cell densities may require lower peptide concentrations. Bacteria were added to the peptide solution on a vortex mixer to assure rapid mixing. Cells treated in the same manner, but without peptide present, served as controls. Following treatment with peptide or diluent, the cells were pelleted, the supernatant removed, and they were then resuspended in 1.25% glutaraldehyde plus 2% formaldehyde in 0.1 M sodium calcodylate (pH 7.4) for 30 min.

Figure 1. A scanning electron micrograph of untreated *E. coli* collected on polycarbonate filters. Bacterial surfaces are relatively smooth, and the cells are not aggregated into clumps.

Figure 2. A higher magnification scanning electron micrograph of untreated bacteria that better illustrates the smooth surface texture of these cells.

Figure 3. A scanning electron micrograph of *E. coli* treated with 100 μM melittin for 15 minutes. Bacteria are aggregated into clumps of cells enmeshed in debris. Some bacteria appear to be joined into larger masses (arrows), and cell surfaces do not appear smooth.

Figure 4. A higher magnification scanning electron micrograph of melittin treated *E. coli*. Distortions and "blebing" of the cell surface are evident. Material connecting adjacent bacteria is also in evidence (arrow).

Figure 5. A scanning electron micrograph of *E. coli* treated with 100 μM hecate-1 for 15 minutes. Bacteria are aggregated into large clumps enmeshed in debris (arrows).

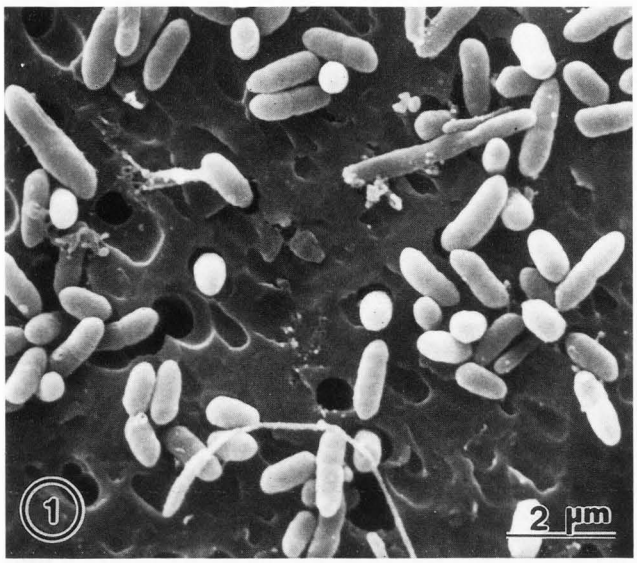
Figure 6. A higher magnification scanning electron micrograph of hecate-1 treated *E. coli*. The cell surfaces are obviously distorted, and the debris surrounding the cells appears to be originating from the cell surfaces (arrows).

Scanning electron microscopy (SEM)

Following the initial fixation, *E. coli* was collected on polycarbonate filters with a pore size of 0.5 μm, post fixed with 1% osmium tetroxide, washed in 0.1 M sodium cacodylate buffer containing 5% sucrose (pH 7.4), dehydrated through an ascending ethanol series to 100% and critical point dried from CO_2. The dried bacteria and filter were mounted on an aluminum stub, sputter coated with gold-palladium, and examined in a Cambridge S-150 SEM (Leica Inc., Deerfield, IL).

Transmission electron microscopy (TEM)

Fixed bacteria were centrifuged into a pellet and washed in 0.1 M sodium cacodylate buffer containing 5% sucrose at pH 7.4. The cells were resuspended in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour, then washed several times in buffer. Following postfixation buffer washes, the pellets were encased in agarose. The hardened agarose containing bacteria was then cut into < 1 mm cubes, dehydrated in an ethanol series, embedded in Spurr's resin, sectioned, stained with uranyl acetate and lead citrate, and examined in either a Philips 410 (Philips Electronic Instruments Co., Mahwah, NJ) or Zeiss EM-109 (Carl Zeiss, Inc., Thornwood, NY) TEM. Alternatively, bacteria were collected on polycarbonate filters and processed through 100% ethanol as described for SEM. Following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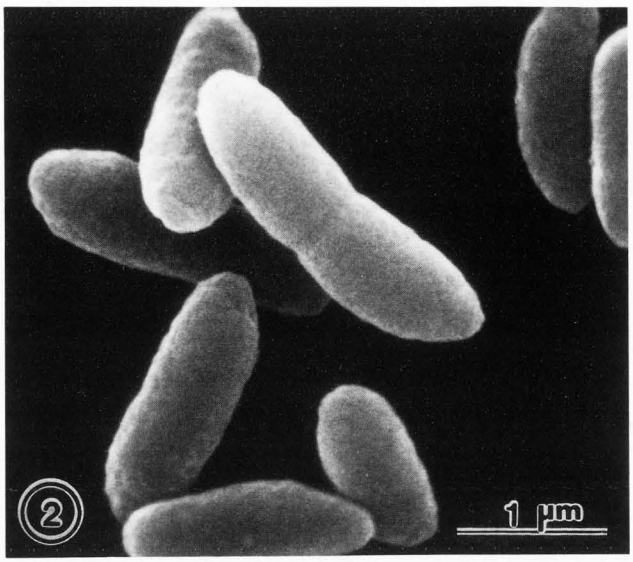
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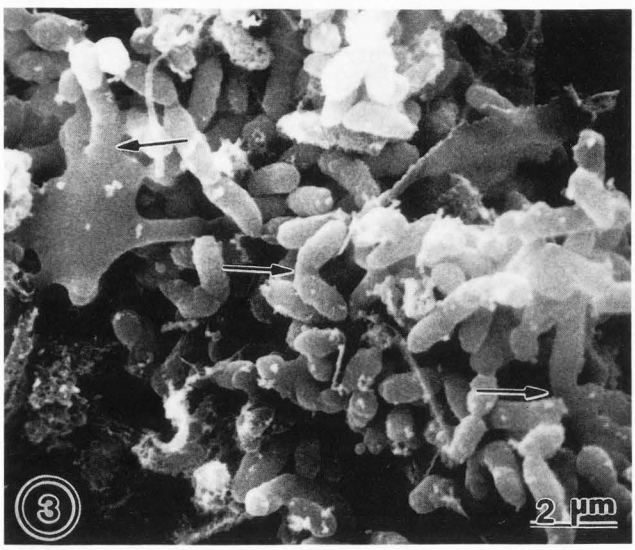
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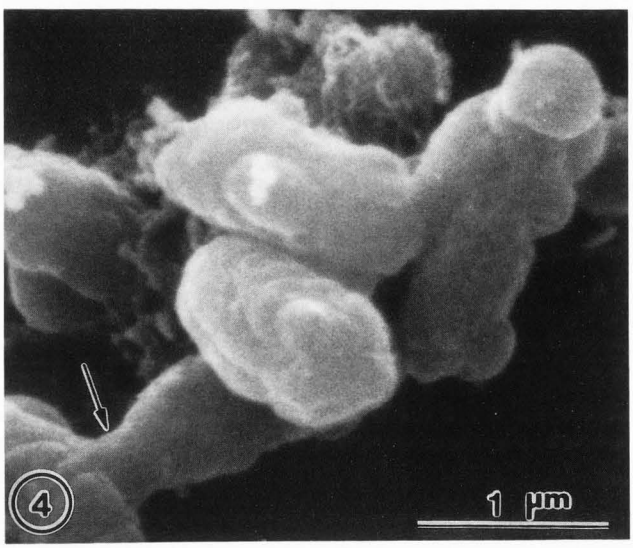
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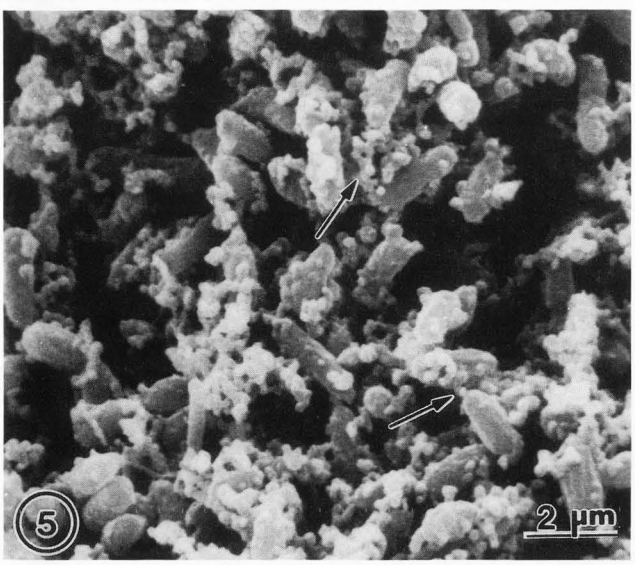
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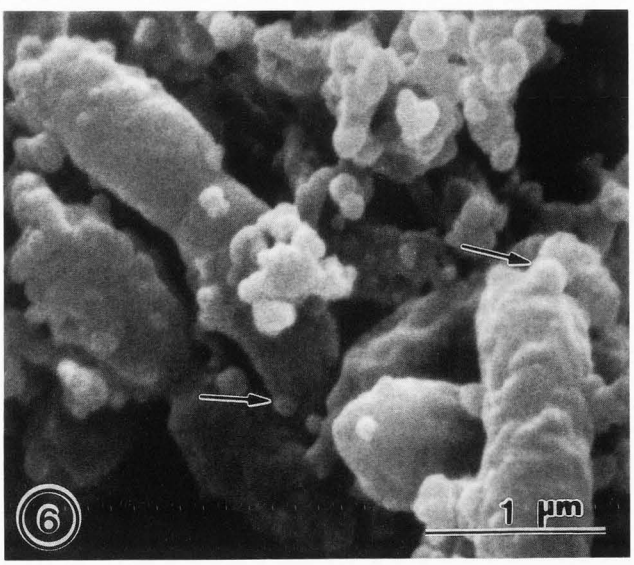
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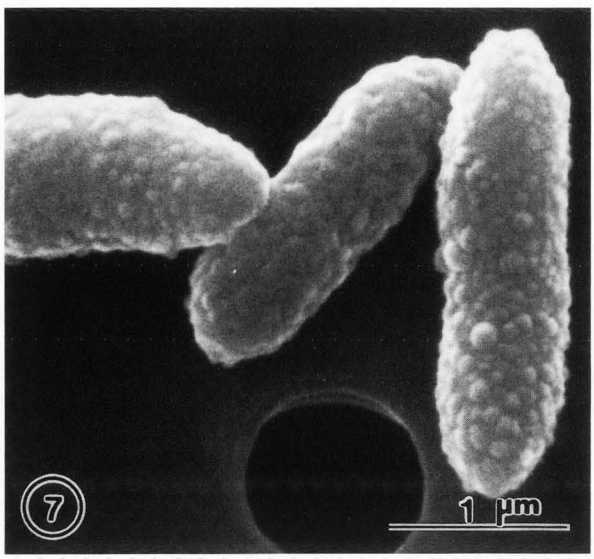

Figure 7. A scanning electron micrograph of melittin treated bacteria occasionally seen in what appears to be a less advanced stage of surface "blebing." The cells also appear less adherent than when the process is more advanced.

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dehydration, the filters were removed from their holders, infiltrated, embedded, sectioned, stained and examined as described above.

Freeze-fracture

Following initial fixation, bacteria were infiltrated in a glycerol series (10-20-30%) and mixed with bakers yeast to make a thick slurry. The slurry was rapidly frozen by plunging into liquid propane at 93K using a Balzers (TFD010) Transfer-Freeze-Device (Fürstentum, Liechtenstein). The frozen cells were then fractured, and platinum (2 nm)- carbon (20 nm) replicas made in a Balzers BAF-400K (Fürstentum Liechtenstein) freeze-fracture apparatus. Replicas were cleaned in sodium hypochlorite, washed, mounted on grids, and examined in the TEM.

Results

Untreated *E. coli*, when observed with the scanning electron microscope, appear smooth, unaggregated and relatively free of extracellular debris (Figs. 1, 2). In both hecate treated and melittin treated samples, the bacteria are clearly aggregated amid a considerable amount of extracellular material (Figs. 3, 5). At higher magnification (Figs. 4, 6), surfaces of treated cells appear badly distorted. Numerous bleb-like protrusions extend from the surface of many cells (Figs. 4, 6), and some cells

Figure 8. A transmission electron micrograph of untreated *E. coli*. Cells are not aggregated and their surfaces appear smooth. Inset (a): A higher magnification of *E. coli* illustrating the cell surface in more detail.

Figure 9. A transmission electron micrograph of untreated, freeze-fractured *E. coli*. The surfaces of the fracture faces of both inner and outer membranes are evident.

Figure 10. A transmission electron micrograph of *E. coli* treated for 15 minutes with 100 μM melittin. The cells are aggregated and electron dense material is evident on their surface. The material appears similar to what is seen in SEM but clearly does not represent a simple blebing of the outer membrane or osmotic rupturing of the cells. Inset (a): Melittin treated *E. coli* at a higher magnification illustrating the cell surface in lore detail.

Figure 11. A transmission electron micrograph of melittin treated, freeze-fractured *E. coli*. A portion of a cross-fractured cell is evident in the upper right corner (asterisk). The inner membrane of the central cell is clearly damaged and surface protrusions that may correspond to the dense staining material evident in thin sections are evident (arrowheads).

Figure 12. A transmission electron micrograph of *E. coli* treated for 15 minutes with 100 μM hecate-1. The cells appear aggregated and electron dense material is evident on their surfaces. The material appears similar to what is seen in SEM but clearly does not represent a simple blebing of the outer membrane or osmotic rupturing of the cells. Inset (a): Hecate treated *E. coli* at higher magnification illustrating the cell surface in more detail.

Figure 13. A transmission electron micrograph of hecate-1 treated, freeze-fractured *E. coli*. Two cross-fractured cells are evident (asterisk). The fracture plane extending through the central cell clearly reveals a severely damaged inner membrane. Material extending from the surface is also evident (arrowhead).

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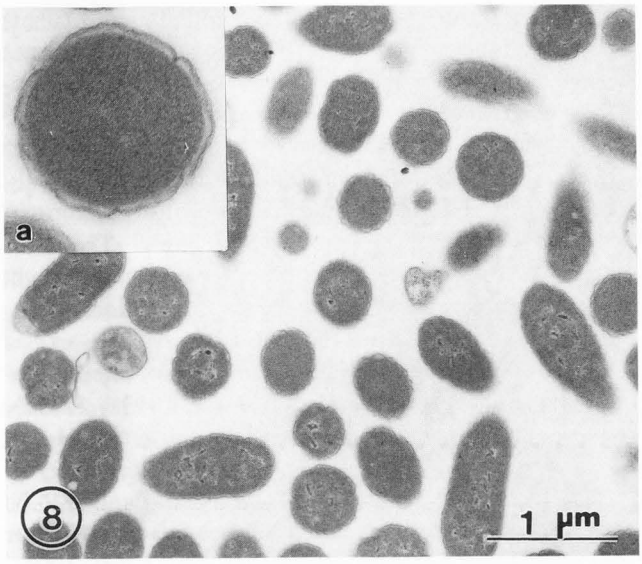
appear to be embedded in a common matrix with their neighbors [Figs. 3, 5 (arrows)]. Occasionally, what appear to be early stages in a dynamic process are seen. Here, the surface blebing is not as pronounced and the cells not as adherent (Fig. 7).

Examination of thin sectioned material with the TEM reveals the apparent absence of the outer membrane and much of the peptidoglycan layer in cells treated with either peptide as opposed to the normal appearance of untreated cells (compare Figs. 8, 8a, 10, 10a, and 12, 12a). Dark masses of material not present in untreated cells (Fig. 8) extend from the damaged cell surfaces and intermingle with similar material from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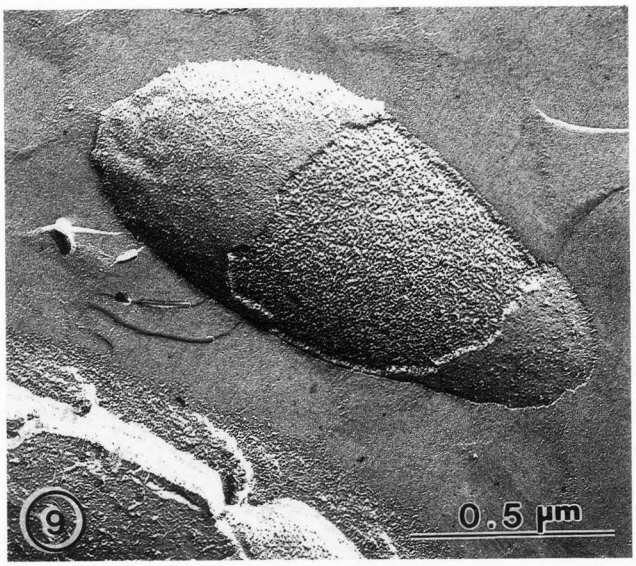
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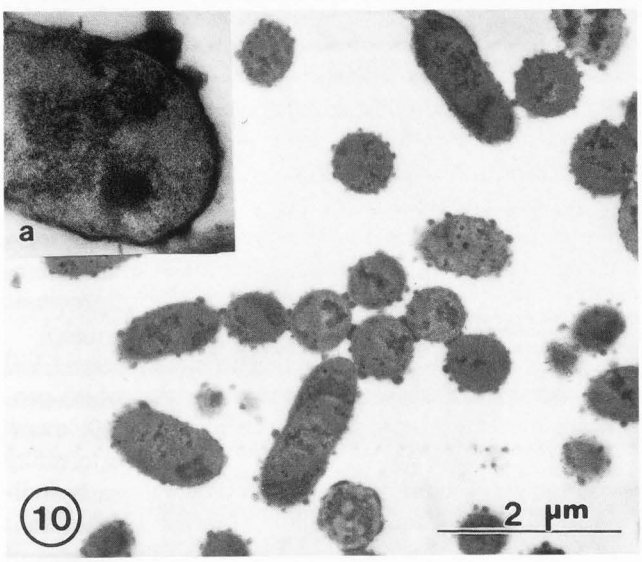
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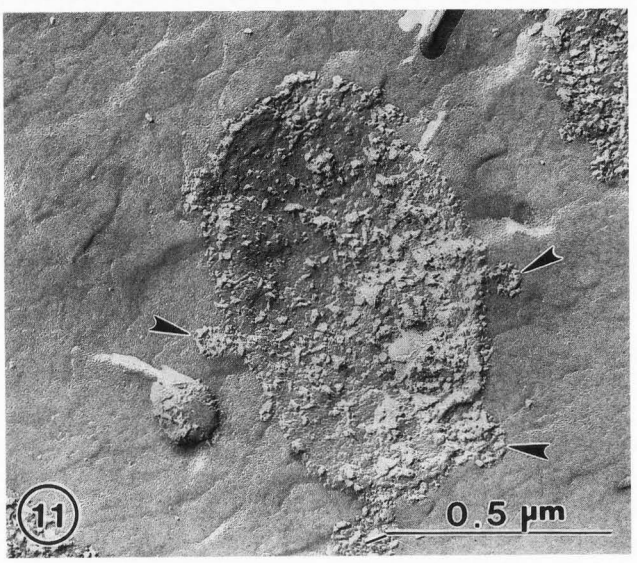
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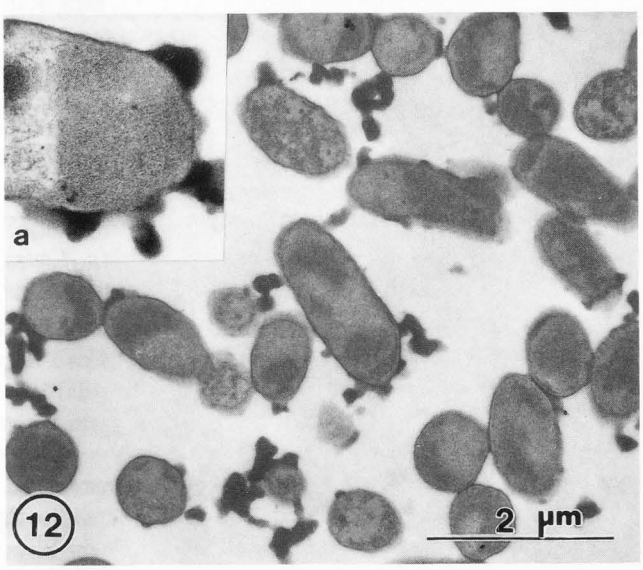
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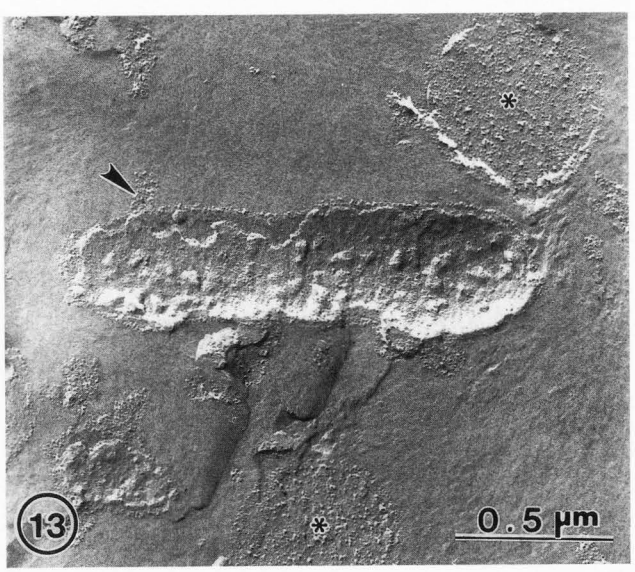
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adjacent cells (Figs. 10 and 12), sometimes enveloping several cells in an amorphous mass of electron dense material.

Freeze-fracture of untreated *E. coli* produce typical profiles of the fracture faces of both inner and outer membranes as well as a clear indication of the peptidoglycan layer between them (Fig. 9). Examination of replicas of bacteria treated with either of the peptides reveals that most cells were cross-fractured, with few fracture planes extending along the membrane face (Figs. 11 and 13). Profiles of the outer membrane or the peptidoglycan cell wall were seen only rarely. Fracture planes along the inner membrane were more common, and often revealed clearly abnormal morphology (compare Figs. 9, 11, and 13). Protrusions from the surface, probably corresponding to the extruded material seen in the thin sections and in SEM, is evident in Figures 11 and 13 (arrows).

Discussion

Both melittin and its 23 amino acid analog hecate-1 appear to have fundamentally similar effects on *E. coli*. From the SEM, it is clear that the bacterial surfaces are profoundly altered during exposure to the peptides. Material appears to be extruded from the bacterial surface to form an adherent matrix holding cells together in large aggregates. The bleb-like protrusions from the bacterial surface seen in SEM appear, upon examination with TEM, to represent the remains of the outer membrane and peptidoglycan layer rather than exuded protoplasm. Transmission electron microscopy confirms the SEM observation that several cells may be enveloped in a mass of extruded material. The staining intensity of extruded material and whether or not cytoplasmic electron dense inclusions (Fig. 10) are present varies regardless of which peptide is used. Electron dense material similar in appearance has been seen on the surface of *E. coli* treated with mammalian defensins (Lehrer *et al.*, 1989). In freeze-fracture preparations, the absence of frequent fractures along the planes of the outer or inner membrane also indicates that these membranes are severely damaged, if not entirely missing. The cytoplasmic contents of the bacteria, however, do not appear radically disturbed, and there is little evidence for the osmotic extrusion of protoplasm. The fate of the peptides during this process is not known, but it would be useful to determine whether or not the peptides are associated with the dense remnants of the outer membrane and cell wall.

Under the conditions described here, either peptide kills virtually 100% of bacteria, but the cells do not appear osmotically ruptured. Membrane damage is massive and cell-wide, rather than localized in discrete regions of the cell surface, but the protoplasmic contents of the cells remain in place. These observations do not support the view that the bacteriostatic action of the peptides on gram negative bacteria involves osmotic instability, resulting from the formation of ion channels or pores. A membrane disintegration mechanism, recently proposed to explain the antibacterial action of cecropin B (Gazit *et al.*, 1994), appears more likely. The interaction of the peptides with the outer membrane might also involve displacement of the divalent cations necessary to maintain its integrity. Such a mechanism has been proposed in the case of the aminoglycoside antibiotic gentamicin (Kadurugamuwa *et al.*, 1993a, 1993b), the polymyxins, and defensins and the antimicrobial peptide cecropin B (Vaara, 1992; Vaara and Vaara, 1994). Destruction of the outer membrane is not thought to be sufficient by itself to cause antimicrobial action (Lehrer *et al.*, 1989; Vaara, 1992; Vaara and Vaara 1994). Other research, using phospholipid membrane model systems and antimicrobial peptides not related to melittin, has also suggested mechanisms for membrane peptide interaction that do not involve pore or channel formation (Steiner *et al.*, 1988; Bechinger and Seelig, 1991; Matsuzaki *et al.*, 1991). Since obvious aggregates of intramembranous particles were not seen in our freeze-fracture micrographs, we have no evidence for a membrane protein aggregation mechanism of membranolysis. It is possible that only the effects of processes that occur very rapidly were observed.

It might be suggested that melittin and hecate-1 kill bacteria by a mechanism different than that which produces the extensively studied (Dempsey, 1990) membranolysis of eukaryotic cells. While unable to refute such a proposition, results presented here clearly indicate that the antibacterial activity of melittin is the result of massive damage to the inner and outer membranes of *E. coli*. Even if the mechanisms are different, they both involve severe damage to cell-surface membranes.

Acknowledgments

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References

Bechinger B, Seelig J (1991) Conformational changes of the phosphatidylcholine headgroup due to membrane dehydration. A ^{2}H-NMR study. Chem Phys

Lipids **58**: 1-5.

Boman HG (1991) Antibacterial peptides: Key components needed in immunity. Cell **65**: 205-207.

Boman HG, Wade D, Boman IA, Wåhlin B, Merrifield RB (1989) Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. FEBS Lett **259**: 103-106.

Dawson CR, Drake AF, Helliwell J, Hider RC (1978) The interaction of bee melittin with lipid bilayer membranes. Biochim Biophys Acta **510**: 75-86.

DeGrado WF, Musso GF, Liber M, Kaiser ET, Kezdy FJ (1981) Kinetics and mechanism of haemolysis induced by melittin and by a synthetic melittin analog. Biophys J **37**: 329-338.

Dempsey CE (1990) The actions of melittin on membranes. Biochim Biophys Acta **1031**: 143-161.

Dufourcq J, Faucon J-F, Fourche G, Dasseux J-L, Le Maire M, Gulik-Krzywicki T (1986) Morphological changes of phosphatidylcholine induced by melittin: Vesicularization, fusion, discoidal particles. Biochim Biophys Acta **859**: 33-48.

Gazit E, Lee W-J, Brey PT, Shai Y (1994) Mode of action of the antibacterial cecropin B2: a spectrofluorometric study. Biochemistry **33**: 10681-10692.

Hui SW, Stewart CM, Cherry RJ (1990) Electron microscopic observation of the aggregation of membrane proteins in human erythrocyte by melittin. Biochim Biophys Acta **1023**: 335-340.

Kadurugamuwa JL, Lam J, Beveridge TJ (1993a) Interaction of gentamicin with the AB and B band lipopolysaccharides of *Pseudomonas aeruginosa* and its possible lethal effect. Antimicrob Agents Chemother **37**: 715-721.

Kadurugamuwa JL, Clarke AJ, Beveridge TJ (1993b) Surface action of gentamicin on *Pseudomonas aeruginosa*. J Bacteriol **175**: 5798-5805.

Katsu T, Kuroko M, Morikawa T, Sanchika K, Fujita Y, Yamamura H, Uda M (1989) Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. Biochim Biophys Acta **983**: 135-141.

Lee JY, Boman A, Chuanxin S, Andersson M, Jörnvall H, Mutt V, Boman HG (1989) Antibacterial peptides from pig intestine: Isolation of a mammalian cecropin. Proc Natl Acad Sci, USA **86**: 9159-9162.

Lehrer RI, Barton A, Daher KA, Harwig SS, Ganz T, Selsted ME (1989) Interaction of human defensins with *Escherichia coli*. J Clin Invest **84**: 553-561.

Maloy WL, Kari UP (1995) Structure-activity studies on magainins and other host defense peptides. Biopolymers (Peptide Science) **37**: 105-122.

Matsuzaki K, Harada M, Funakoshi S, Fujii N, Miyajima K (1991) Physicochemical determinants for interactions of magainins 1 and 2 with acidic lipid bilayers. Biochim Biophys Acta **1063**: 162-170.

Steiner H, Andreu D, Merrifield RB (1988) Binding and action of cecropin and cecropin analogues: Antibacterial peptides from insects. Biochim Biophys Acta **939**: 260-266.

Tosteson MT, Auld DS, Tosteson DC (1989) Voltage-gated channels formed in bilayers by positively charged segment of the Na-channel polypeptide. Proc Natl Acad Sci, USA **86**: 707-710.

Tosteson MT, Alvarez O, Hubbell W, Bieganski RM, Attenbach C, Caporales LH, Levy JJ, Nutt RF, Rosenblatt M, Tosteson DC (1990) Primary structure of peptides and ion channels. Role of amino acid side chains in voltage gating of melittin channels. Biophys J **58**: 1367-1375.

Vaara M (1992) Agents that increase the permeability of the outer membrane. Microbiol Rev **56**: 395-411.

Vaara M, Vaara T (1994) Ability of cecropin B to penetrate the enterobacterial outer membrane. Antimicrob Agents Chemother **38**: 2498-2501.

Weaver AJ, Kemple MD, Brauner JW, Mendelsohn R, Prendergast FG (1992) Fluorescence, CD, attenuated total reflectance (ATR) FTIR, and ^{13}C NMR characterization of the structure and dynamics of synthetic melittin and melittin analogs in lipid environments. Biochemistry **31**: 1301-1313.

Zasloff M (1992) Antibiotic peptides as mediators of innate immunity. Curr Opin Immunol **4**: 3-7.

Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers.**