Interaction of Human Defensins with *Escherichia coli*

Mechanism of Bactericidal Activity

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Abstract

Defensins are small, cysteine-rich antimicrobial peptides that are abundant in human, rabbit, and guinea pig neutrophils (PMN). Three defensins (human neutrophil peptide defensin [HNP]-1, HNP-2, and HNP-3) constitute between 30 and 50% of the total protein in azurophil granules of human PMN. We examined the mechanism of HNP-mediated bactericidal activity against *Escherichia coli* ML-35 (i^-, y^-, z^+) and its pBR322-transformed derivative, *E. coli* ML-35p. Under conditions that supported bactericidal activity, HNP-1 sequentially permeabilized the outer membrane (OM) and inner membrane (IM) of *E. coli*. Coincident with these events, bacterial synthesis of DNA, RNA, and protein ceased and the colony count fell. Although these events were closely coupled under standard assay conditions, OM permeabilization was partially dissociated from IM permeabilization when experiments were performed with *E. coli* that had been plasmolyzed by mannitol. Under such conditions, the rate and extent of bacterial death more closely paralleled loss of IM integrity than OM permeabilization. Electron microscopy of *E. coli* that had been killed by defensins revealed the presence of striking electron-dense deposits in the periplasmic space and affixed to the OM. Overall, these studies show that HNP-mediated bactericidal activity against *E. coli* ML-35 is associated with sequential permeabilization of the OM and IM, and that inner membrane permeabilization appears to be the lethal event.

Introduction

The ability of PMN to ingest and kill microorganisms contributes substantially to host defenses against infection. PMNs possess at least two general antimicrobial mechanisms. One of these, often designated "oxygen dependent," results from the PMN's postphagocytic production of O_2^- (superoxide) that is subsequently converted to more potent oxidants such as H_2O_2, hydroxyl radical, hypochlorous acid, and chloramines. These stronger oxidants are likely to be the actual effectors of oxygen-dependent microbicidal activity (comprehensively reviewed in reference 1).

The other general microbicidal mechanism, usually called "oxygen independent," results when PMN translocate antimicrobial proteins from cytoplasmic granules to their phagocytic vacuoles. Among these antimicrobial components are defensins (2, 3), cathepsin G (4–6), cationic antimicrobial peptide (CAP)-37 (7), and three similar or identical proteins, known variously as bactericidal/permeability-increasing protein (B/PI)[1] (8, 9), CAP-57 (7), and bactericidal protein (10).

Defensins are small (M_r 3,500–4,000), carbohydrate-free homologous peptides that exist in human (2, 11), rabbit (12), and guinea pig (13) PMN. The human PMN's three defensins, human neutrophil peptide defensin (HNP)-1, HNP-2, and HNP-3, have identical primary sequences, except for their respective amino terminal residues (3). Human and animal defensins exert in vitro activity against a broad range of gram-positive and -negative bacteria (2, 14), fungi (15, 16), and enveloped viruses (17, 18). We undertook these experiments to ascertain the bactericidal mechanism of defensins against a susceptible gram-negative bacterium, *Escherichia coli* ML-35.

Methods

Organisms. E. coli ML-35, a lactose permease–deficient strain with constitutive cytoplasmic β-galactosidase activity (i^-, y^-, z^+), was obtained from Professor S. C. Rittenberg of UCLA. *E. coli* ML-35p was constructed by transforming *E. coli* ML-35 with pBR-322 to introduce a periplasmic, plasmid-encoded β-lactamase as reported elsewhere (19). *E. coli* ML-35 was maintained on trypticase soy agar plates and *E. coli* ML-35p was passaged on similar plates that periodically contained 100 μg/ml of ampicillin. Organisms were picked from a single colony and incubated in trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, MD) for 18 h at 37°C to provide stationary phase test organisms. These cultures were washed two or three times with 10 mM sodium phosphate buffer, pH 7.4 (NAPB), then adjusted to an OD_{620} of 0.35 ($\sim 1 \times 10^8$ CFU/ml) and kept on ice until used.

Peptides and reagents. Defensins were prepared from human peripheral blood leukocytes as previously described (2). Experiments were done with either purified HNP-1 or a mixture of two or three defensins, as described in the text. Working stock solutions of defensins were usually prepared at 1 mg/ml in 0.01% acetic acid. Other reagents and their suppliers were: 7-[(thienyl-2-acetamido)-3-(2-(4-*N,N*-dimethylaminophenylazo)-pyridinium-methyl)-3-cephem-4-carboxylic acid] (PADAC), Calbiochem-Behring Corp., La Jolla, CA; *o*-nitrophenyl-β-D-galactoside (ONPG) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), Sigma Chemical Co., St. Louis, MO; and

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1. *Abbreviations used in this paper:* B/PI, bactericidal/permeability-increasing protein; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; HNP, human neutrophil peptide defensin; IM, inner membrane; NAPB, 10 mM sodium phosphate buffer; OM, outer membrane; ONPG, *o*-nitrophenyl-β-D-galactoside; PADAC, 7-[(thienyl-2-acetamido)-3-(2-(4-*N,N*-dimethylaminophenylazo)-pyridinium-methyl)-3-cephem-4-carboxylic acid]; TSB, trypticase soy broth.

glutaraldehyde (8% aqueous, E.M. grade), Polysciences, Warrington, PA.

Membrane permeabilization. To measure inner membrane (IM) permeabilization, washed stationary phase (18 h) *E. coli* ML-35 were added to a cuvette that contained 1.67 mM ONPG in NAPB-TSB, a medium that contained NAPB, pH 7.4, and a 1:100 dilution of full-strength TSB. When we assessed IM and outer membrane (OM) permeabilization concurrently, *E. coli* ML-35p replaced *E. coli* ML-35, and both PADAC, a β-lactamase substrate, and 1.67 mM ONPG were present in the cuvettes. In most cases we measured OD every 1 or 2 min at 660, 570 (or 595 nm), and 400 nm (ONPG) as previously described (19). All spectrophotometric studies were performed in a spectrophotometer (model DU-8; Beckman Instruments, Inc., Palo Alto, CA) with a Peltier temperature controller. Cuvettes contained 0.6–3.0 ml, depending on the number and volume of samples to be removed during the incubation.

In some experiments we used a modified version of the previously described multiple wavelength assay, and expressed OM and IM permeabilization relative to that of fully permeabilized bacteria. The essential elements of this more quantitative procedure will be described below. Data reduction was performed with Lotus 1-2-3 software (Lotus Development Corp., Cambridge, MA).

Modified spectrophotometric assay. The effects of defensins on permeability of the OM and IM of *E. coli* ML-35p was determined as follows. Our primary data consisted of sets of measurements of OD at 660 nm [1], 570 or 595 nm [2], and 400 nm [3] taken at regular intervals, usually 1 or 2 min. In the formulas that follow, the initial readings at these wavelengths are denoted by a subscript, e.g., $[1_0]$ or $[2_0]$. Although the following formulas also apply if different wavelengths are chosen, the numerical values of k_1, k_2, k_3, and k_4 will change.

1. Measure OD_{660}
2. Measure OD_{595}
3. Measure OD_{400}
4. Calculate ΔOD_{660} from [1];
5. Calculate ΔOD_{595} from [2], multiply by -1 to change the sign,
6. $([5] + k_1[4])$ $(k_1 = 1.2103)$
7. $k_2[6]$. $(k_2 = 1.1218)$
8. $\Sigma[7]$.
9. $([2_0] - k_1[1_0]) - [8]$.
10. $[7] \times ([2_0] - k_1[1_0])/[9]$.
11. Calculate ΔOD_{400} from [3].
12. $[11] - k_3[6]$ $(k_3 = 0.35659)$
13. $[12] - k_4[4]$ $(k_4 = 2.5230)$
14. $([11]/k_5) \times 100$ (β-galactosidase).
15. $[10]/k_6] \times 100$ (β-lactamase).

Explanation: the primary data sets [1], [2], and [3] are used to calculate ΔOD_{660} [4], ΔOD_{595} [5], and ΔOD_{400} [11]. All OD changes that occurred at 660 nm are attributable to alterations in light scattering by the bacteria. Although most of the OD change at 570 or 595 nm is due to PADAC hydrolysis (reflecting OM permeability), a small component arises from changes in light scattering and is corrected for in [6].

After complete hydrolysis of PADAC by β-lactamase, some residual light absorption persists at 595 nm, attributable to PADAC's hydrolysis product. Its extent can be measured by allowing *E. coli* β-lactamase to hydrolyze PADAC to completion, and used to define a constant, k_2, that equals the initial OD_{595}/(initial OD_{595} − final OD_{595}) and is used in [7].

Cumulating the readings in [7] provides the total amount of PADAC consumed [8]. By subtracting the amount of PADAC consumed [8] from the amount initially present $([2_0] - k_1 [1_0])$, the residual PADAC concentration is calculated [9]. Because PADAC hydrolysis in this system was first order with respect to substrate concentration for PADAC concentrations up to 100 μM (data not shown), we normalized the instantaneous rates of PADAC consumption [7] to the concentration of substrate that was present at the outset of the reaction. This is shown in [10], which multiplies the corrected instantaneous rate[2] of PADAC hydrolysis [7] by a factor equal to the initial PADAC concentration $([2_0] - k_1[1_0])$ divided by the residual PADAC concentration [9].

The rate of ONPG hydrolysis is calculated more simply by correcting the measured DOD_{400} [11] for contributions made by PADAC's hydrolysis product [12] and for bacterial light scattering [13]. The turbidity constants k_1 and k_4 were determined by scanning *E. coli* (1×10^7 CFU/ml) at 660, 595, and 400 nm. The factor, k_3, indicating the contribution of PADAC hydrolysis to absorbance at 400 nm, was determined experimentally as previously described by monitoring the change in absorbance at both wavelengths when PADAC was hydrolyzed by purified β-galactosidase (19).

To determine the maximal rate of ONPG $[k_5]$ and PADAC $[k_6]$ hydrolysis by fully permeabilized cells, we removed samples of defensin-treated bacteria from the cuvettes, placed them in a melting ice bath, and applied three 15-s cycles of sonication at 70% power with the small probe of a Bronson Biosonic IV sonicator (VWR Scientific Inc., San Francisco, CA). These sonicates were incubated with ONPG and PADAC. In most instances the total enzyme activity in defensin-treated cells remained constant during the assay (Fig. 5) and a sample removed at 20 min was adequate.

Before deciding on sonication to fully permeabilize the bacteria, we also tried toluene and a variety of surface-active compounds, none of which were better than sonication in our hands (data not shown). To express IM permeabilization as a percent of maximal, we divided the corrected ΔOD_{400} [13] by the reaction rate recorded by sonicated cells $[k_5]$ as shown in [14]. OM permeabilization was calculated in an analogous manner, by dividing the fully corrected rate of PADAC hydrolysis [10] by the reaction rate measured in sonicated cells (k_6), as in [15].

Isotope incorporation. Aqueous solutions of uniformly labeled L-[^{14}C]leucine (342 mCi/mmol), [^{3}H]methyl-uridine (29 Ci/mmol), and [^{3}H]methyl-thymidine (87 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL) and their specific activities were adjusted by appropriate additions of stable compound. Some isotope studies were performed in cuvettes that contained NAPB-TSB and 1.67 mM ONPG to allow the inhibition of macromolecular synthesis to be timed relative to the onset of IM permeabilization.

At intervals, 25-μl samples were removed to measure isotope incorporation. These were mixed with 50 μl of 2% Triton X-100 in NAPB and diluted with 2.5 ml of cold 10% TCA. Duplicate 1-ml aliquots were deposited on GFC/C glass fiber filters (Whatman LabSales, Hillsboro, OR). These were washed three times with 1 ml of cold 5% TCA and once with methanol and placed in minivials containing 5 ml of ACS counting fluid (Amersham Corp.). Radioactivity was measured with an LS-100 liquid scintillation counter (Beckman Instrument, Inc., Palo Alto, CA).

Electron microscopy. Four 10-ml samples that contained *E. coli* ML-35p (5×10^7 CFU/ml) in NAPB-TSB were preincubated for 30 min at 37°C. To three of these samples we added defensins (100 μg/ml of an equimolar mixture of HNP-1 and -2), while the fourth, a control, received an equivalent vol of 0.01% acetic acid (80 μM, final concentration). A pilot sample containing PADAC, whose composition was otherwise identical to the samples prepared for electron microscopy, was monitored to time the onset of OM permeabilization. Samples for electron microscopy were harvested after 20, 30, and 60 min. After removing a small portion of these samples for CFU/ml measurements, the remainder was centrifuged for 10 min at 2,800 *g*. Pellets were resuspended and fixed for 1 h in 2.5% glutaraldehyde/0.75 M cacodylate buffer, pH 7.4, and postfixed for an additional h with 1% OsO_4 in

2. In this description the rate formulas were written in a time-independent manner because time intervals between measurements were equal. If this were not so, [4], [5], and [11] would have been divided by ΔT, as would k_5 and k_6. The authors will provide interested readers with a copy of this program, written for Lotus 1-2-3, on request.

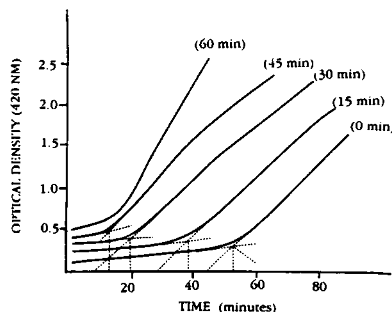


Figure 1. Defensin-mediated IM permeabilization: effect of preincubation. An 18-h culture of *E. coli* ML-35 was washed, suspended at 1.5×10^7 CFU/ml, and preincubated in NAPB-TSB containing 1.67 mM ONPG for 0, 15, 30, 45, or 60 min before the addition of 50 μg/ml HNP 1-3 (a mixture of HNP-1, HNP-2, and HNP-3 in a 1:1:0.5 molar ratio). The cuvettes were monitored at 60-s intervals at 420 nm to detect ONPG hydrolysis, a manifestation of inner membrane permeabilization. Preincubation times are shown in parentheses. From the data shown here we calculated the lag time before IM permeabilization by extending lines from the baseline and maximal slopes and dropping a perpendicular line from their intersection as illustrated by the interrupted lines.

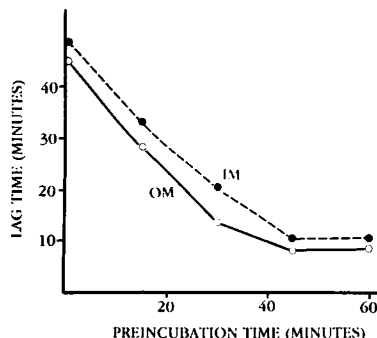


Figure 2. Kinetics of OM and IM permeabilization. An 18-h culture of *E. coli* ML-35p was washed and suspended at 1 × 107 CFU/ml in cuvettes that contained NAPB-TSB and either ONPG (1.67 mM) or PADAC (20 μM). After these had been preincubated at 37°C for 0, 15, 30, 45, or 60 min, an equimolar mixture of HNP-1 and HNP-2 (final concentration 100 μM/ml) was added and the time required until the onset of enhanced PADAC (○) or ONPG (●) hydrolysis was measured to indicate OM (○) and IM (●) permeabilization, respectively.

PBS. Specimens were embedded in Spurr, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-100 CX microscope.

Results

IM permeabilization. Intact stationary phase *E. coli* ML-35 exposed to HNP-1 in NAPB remained cryptic for β-galactosidase for > 90 min (data not shown). In contrast, when these organisms were exposed to defensins in a nutrient-containing buffer, NAPB-TSB, ONPG hydrolysis occurred, signifying that the IM had become permeable (Fig. 1).

IM permeabilization occurred after a lag that varied from 12 to ~ 50 min. The lag times were longest when stationary phase bacteria that had not been preincubated in NAPB-TSB were tested. When tests were performed with bacteria that had been preincubated in NAPB-TSB, the lag times were shortened by 1 min for each min of preincubation until a lag of 12–15 min, evidently a limiting value, was reached. When we substituted midlogarithmic phase *E. coli* for stationary phase organisms in this assay, the lag preceding IM permeabilization was also between 12 and 15 min (data not shown). HNP-2, but not HNP-3 (100 μg/ml), also caused IM permeabilization under similar experimental conditions (data not shown).

OM permeabilization. We examined the effects of human defensins on OM permeability in *E. coli* ML-35 that had been transformed with pBR322. Such target cells contained a periplasmic β-lactamase that under our experimental conditions was substantially (92–96%) cryptic for PADAC unless OM permeabilization supervened, an event attended by an abruptly increased rate of PADAC hydrolysis (19).

Fig. 2 compares the effects of preincubation on the duration of the lag preceding defensin-mediated IM and OM permeabilization in *E. coli* ML-35p. Note that both lags responded in parallel, shortening from a maximal value of 45–49 min for nonpreincubated, stationary phase organisms to as little as 8–10 min for organisms that had been incubated for 45–60 min. HNP-2, but not HNP-3, also induced OM permeabilization under these conditions (data not shown).

Macromolecular synthesis. We determined the effects of defensins on macromolecular synthesis in *E. coli* ML-35 that had been preincubated for 30 min in NAPB-TSB. [^{14}C]-Leucine and either [^{3}H]uridine or [^{3}H]thymidine were then added, followed immediately by an addition of defensins or an equivalent vol of 0.01% acetic acid (controls).

Although defensin-treated and control *E. coli* ML-35 displayed equivalent rates of DNA, RNA, and protein synthesis for the first 10–15 min, thereafter macromolecular synthesis ceased completely and concomitantly in defensin-treated bacteria (Fig. 3). The temporal relationships between IM permeabilization, protein synthesis, and loss of colony forming potential are shown in Fig. 4. Note that loss of bacterial viability coincided temporally with the loss of IM integrity, as reflected by ONPG hydrolysis.

The uppermost A_{420} plateau shown in the left portion of the figure is an artefact arising from the spectrophotometer's inability to measure higher ODs. Note also that whereas only two-thirds of the bacteria lost viability (colony forming potential), there was virtually complete cessation of macromolecular synthesis. This observation suggests that sublethal concentrations of HNP may yet exert bacteriostatic effects.

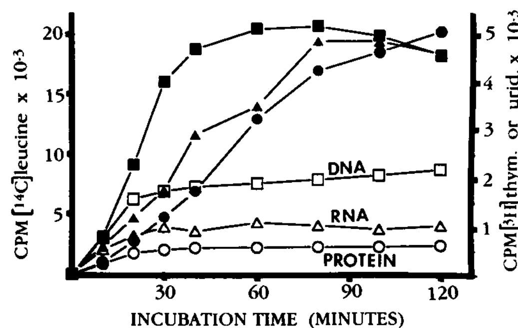


Figure 3. Effects of defensins on macromolecular synthesis. After stationary phase *E. coli* ML-35 (1×10^7 CFU/ml) were preincubated for 30 min in NAPB-TSB at 37°C, concentrated 10-fold, and placed on ice. These bacteria (60 μl) were diluted 10-fold into NAPB-TSB that contained 50 μg/ml of HNP-1,2 (in equimolar amounts). In addition, the assay media contained [^{14}C]leucine (6 μCi, 342 μCi/μmol) and either [^{3}H]uridine (6 μCi, 145 μCi/μmol) or [^{3}H]thymidine (60 μCi, 8.7 mCi/μmol). Duplicate aliquots were removed from each sample and processed as described in the text. The two sets of data for [^{14}C]leucine incorporation were in close agreement and are combined in this figure. *Closed symbols,* controls; *open symbols,* defensin-treated *E. coli* ML-35; *squares,* [^{3}H]thymidine; *triangles,* [^{3}H]-uridine; *circles,* [^{14}C]leucine.

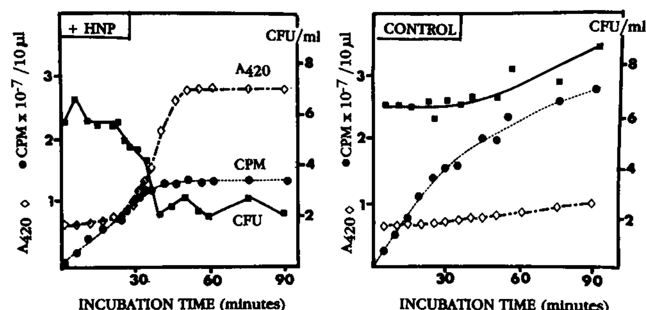


Figure 4. Effects of defensins on protein synthesis, IM permeabilization, and loss of viability. A cuvette containing ~ 5×10^7 *E. coli* ML-35 in 1 ml of NAPB-TSB with 1.67 mM ONPG was preincubated at 37°C for 30 min. Then 0.1 ml of [^{14}C]leucine (final concentration 1 μCi/ml) and 100 μg/ml HNP-1 were added and serial aliquots were removed to measure isotope incorporation and CFU/ml while the cuvette was monitored for ONPG hydrolysis. The plateau in A_{420} arises from the spectrophotometer's insensitivity to higher ODs, rather than from termination of ONPG hydrolysis.

Effects on enzyme synthesis. We also examined the effects of HNP-1 on synthesis of periplasmic β-lactamase and cytoplasmic β-galactosidase by *E. coli* ML-35p. As shown in Fig. 5, control bacteria incubated in defensin-free NAPB-TSB synthesized both enzymes in ever-increasing amounts, in proportion to their increasing numbers. In contrast, no net synthesis of either enzyme occurred in the defensin-treated *E. coli.*

OM permeabilization. We investigated the effects of HNP on the OM of *E. coli* with a procedure that permitted analysis of IM permeabilization in the same sample at the same time (19). Fig. 6 shows a representative experiment performed with bacteria that had been preincubated for 30 min in NAPB-TSB before addition of HNP-1. Note that the onset of OM and IM permeability occurred ~ 15 min after the defensins had been added.

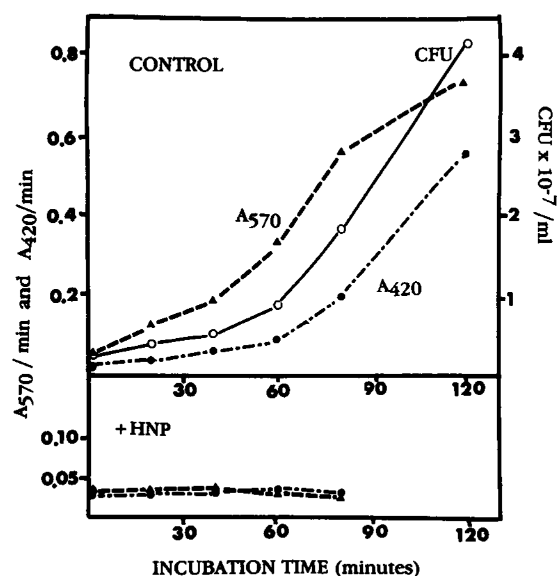


Figure 5. Effects of HNP-1 on enzyme content of *E. coli* ML-35p. *E. coli* ML-35p, ~ 2×10^6 CFU/ml, was preincubated in NAPB-TSB with (*bottom*) or without (*top*) 50 μg/ml of HNP-1. At intervals, samples were removed for colony count measurements and to measure total enzyme activity in bacterial sonicates.

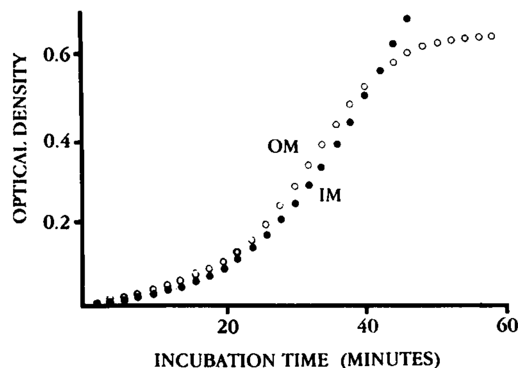


Figure 6. Defensin-mediated OM and IM permeabilization. As 18-h culture of *E. coli* ML-35p (1×10^7 CFU/ml) was preincubated in NAPB-TSB with 1.67 mM ONPG and 20 μM PADAC. After 30 min HNP 1,2 (50 μg/ml) was added and the cuvette was monitored at 570 and 400 nm for an additional 60 min to monitor permeabilization of the OM (*open circles*) and IM (*solid circles*).

Although this method allowed us to delineate the onset of OM and IM permeabilization, it provided little information about the extent or duration of these events. For these reasons, the assay was modified as described in Methods. When we used this modified procedure, OM permeabilization appeared biphasic, with a relatively small early component and a much larger secondary component (Fig. 7). The secondary phase of OM permeabilization coincided precisely in timing and relative extent with permeabilization of the IM. Fig. 7 also confirms that periplasmic β-lactamase was substantially but incompletely cryptic at the outset of the measurement.

Dissociation of OM and IM permeabilization. Although OM and IM permeabilization were tightly coupled in time under our standard test conditions in NAPB-TSB, we could partially dissociate them by conducting the experiment in NAPB-TSB that contained a high concentration (0.5–0.6 M) of mannitol, an osmolyte. Although this dissociation was also evident when the experiments were analyzed according to our simpler, published (19) procedure (Fig. 8), it was even more distinct when the experiments were analyzed by the more

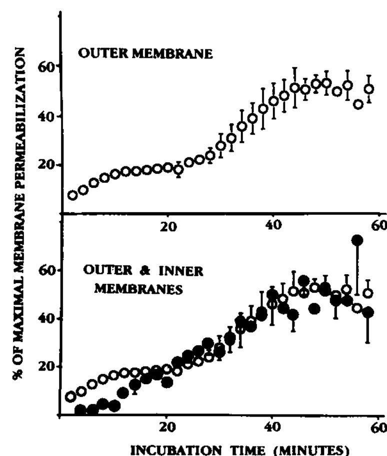


Figure 7. Fine kinetics of defensin-mediated OM and IM permeabilization. An overnight (18-h) culture of *E. coli* ML-35p (2×10^6 CFU/ml) was preincubated for 30 min in NAPB-TSB that contained 44 μM PADAC and 1.67 mM ONPG. HNP-1 (50 μg/ml) was added and readings were taken at 2-min intervals at 660, 570, and 400 nm. *Open circles,* corrected rates of PADAC hydrolysis; *solid circles,* ONPG hydrolysis. *Top,* OM permeabilization; *bottom,* OM and IM permeabilization. Data (mean and SD) from two independent but virtually identical experiments have been combined.

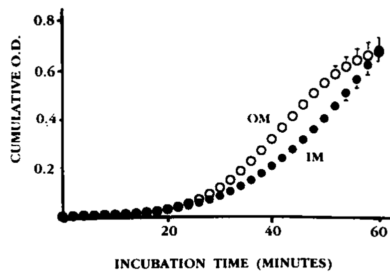


Figure 8. Partial dissociation of OM and IM permeabilization by mannitol. *E. coli* ML-35p (1×10^7 CFU/ml) was preincubated for 30 min at 37°C in NAPB-TSB that contained ONPG, PADAC, and 0.52 M mannitol. HNP-1 (50 μg/ml) was added and the reaction was followed at 570 and 400 nm. Data from two identical experiments, performed on different days, have been combined. *Open circles,* hydrolysis of PADAC (OM); *closed circles,* hydrolysis of ONPG (IM).

quantitative approach described in this report. Unlike our findings in NAPB-TSB (Fig. 7), HNP-induced OM permeabilization proceeded with unimodal kinetics and went to completion when the target bacteria had been plasmolyzed with mannitol (Fig. 9). Under these conditions, IM permeabilization only progressed to ~ 50% of maximum when results were corrected for the slow but progressive inhibition of β-galactosidase by HNP-1 (denoted by solid triangles in the figure). The decreased CFU/ml and the extent of IM permeabilization were very similar, suggesting that IM perforation rather than OM permeabilization was the lethal event.

We were unsuccessful in numerous attempts to fully dissociate OM and IM permeabilization by altering the incubation temperature or by making variously timed additions of CCCP, Ca^{2+}, or chloramphenicol. Although each of these additions could protect the bacterial target cells from defensin-mediated IM permeabilization, they also protected them well from OM permeabilization (data not shown).

Morphological changes. HNP-treated stationary phase *E. coli* that had been exposed to HNP-1 for 60 min showed several remarkable changes in their electron microscopic appearance (Fig. 10). Many bacteria demonstrated a marked accumulation of electron-dense material in the periplasmic space and on the external face of their outer membranes. No such changes were noted in samples that had been fixed after 20

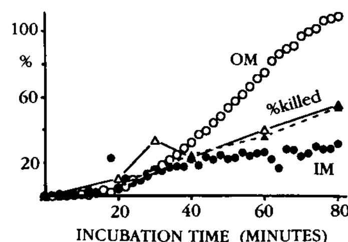


Figure 9. Effect of HNP-1 on membrane permeabilization and bacterial viability in mannitol. *E. coli* ML-35p (2×10^6 CFU/ml) was preincubated for 30 min in NAPB-TSB that contained 1.67 mM ONPG, 90 μM PADAC, and 0.5 M mannitol and then exposed to 50 μg/ml HNP-1. OD measurements were obtained every 2 min at 660, 595, and 400 nm. Aliquots were removed at 20, 40, 60, and 80 min and sonicated to measure the enzyme rates in fully permeabilized cells. IM (●) and OM (○) permeabilization is graphed relative to rates in permeabilized cells removed after 20 min. Although the maximal rate of PADAC hydrolysis by HNP-treated bacteria remained stable during the assay, ONPG hydrolysis by sonicates declined slowly and linearly by 0.56% min^{-1} between 20 and 80 min. Consequently, IM permeabilization is also expressed relative to sonicates prepared at 20, 40, 60, and 80 min (▲). △, Colony counts. In this experiment $k_5 = 0.0268$ min^{-1} and $k_6 = 0.1460$ min^{-1}.

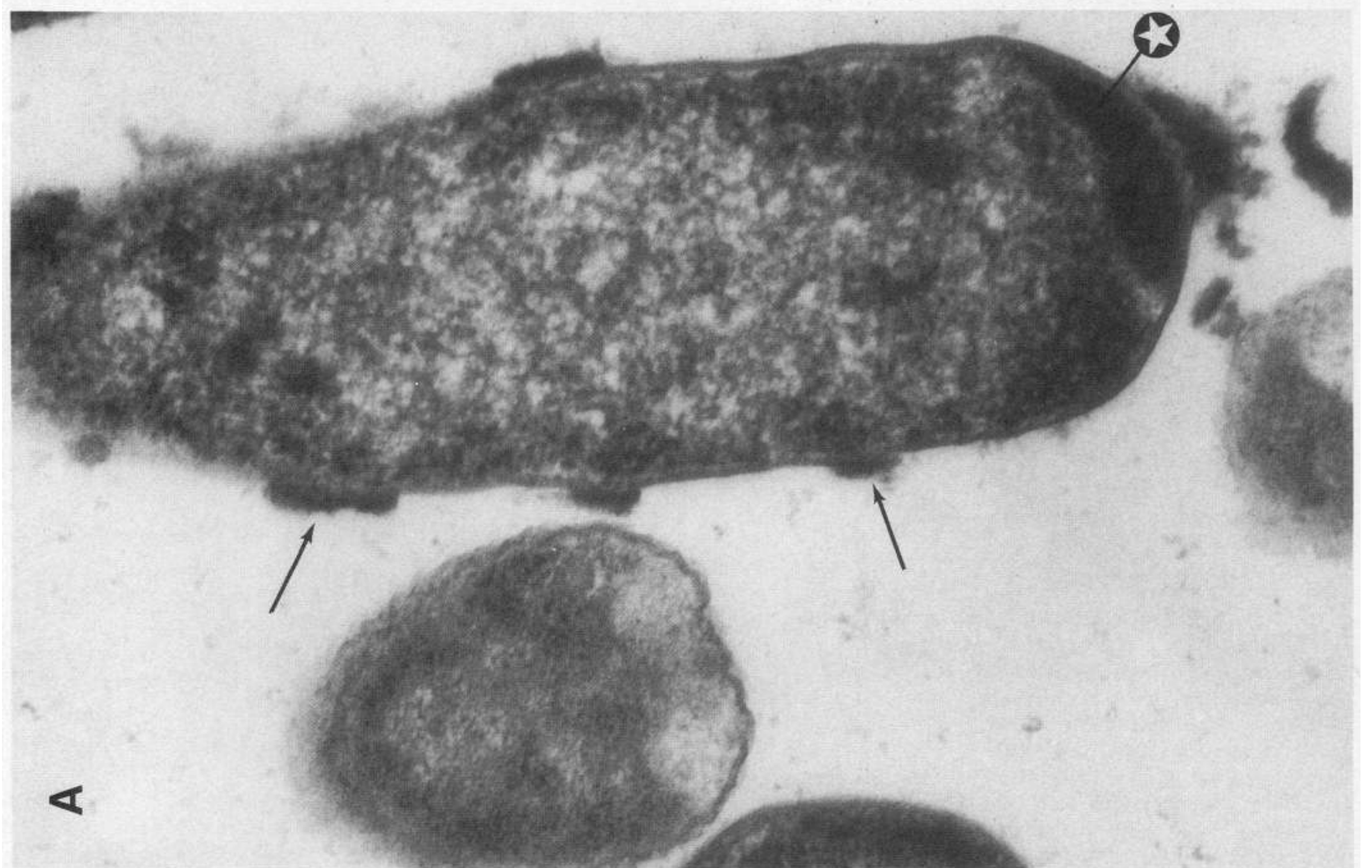
min of exposure to HNP, and only a few organisms with these changes were evident in the samples fixed after 30 min. Colony counts obtained on aliquots of the 20-, 30-, and 60-min samples before fixation showed 33, 50, and 70% reductions, respectively, suggesting that appearance of these structures followed rather than caused loss of bacterial viability. Indeed, in parallel studies of defensin binding we have noted that massive uptake and accumulation of HNP-1 by *E. coli* ML-35 occurs after establishment of OM/IM permeability (Lehrer et al., unpublished observations). Consequently, posthumously bound defensins may contribute to the striking accretions (funereal warts?) shown in Fig. 9.

Discussion

We analyzed the bactericidal mechanism of defensins against a susceptible strain of *E. coli* by establishing the chronology and order of four major events: OM permeabilization, IM permeabilization, inhibition of macromolecular synthesis, and loss of colony forming potential. To do so, we developed a procedure that allowed these events to be monitored concurrently in a single reaction vessel, in this case a cuvette. Although all four events were virtually simultaneous when assays were run in a nutrient-supplemented dilute buffer, NAPB-TSB, they were partially dissociated when we performed the experiments with bacterial targets that had been plasmolyzed with mannitol. Under these conditions, defensin-mediated bactericidal activity was correlated with permeabilization of the bacterial IM. Although defensin-induced IM permeabilization appeared to require antecedent OM damage, the partial dissociation of OM and IM permeabilization by mannitol suggests that the outer membrane lesion is insufficient, by itself, to cause microbial death.

Since the outer membrane of *E. coli* contains porins that normally exclude hydrophilic molecules larger than 700 D from passage into the periplasmic space, the ability of defensins (M_r 3,500–4,000) to cause OM damage may bypass this exclusionary mechanism. We have observed that defensin-treated, but not native *E. coli* ML-35 bacteria can be spheroplasted by lysozyme (Lehrer et al., unpublished observations). Since lysozyme molecules are considerably larger than defensins, it is logical to conclude that defensin-mediated OM permeabilization also affords defensins access to the periplasmic space and IM. Sawyer et al. recently reported that a rabbit defensin, NP-1, can permeabilize the OM of *Pseudomonas aeruginosa* (20).

Our use of the hydrophilic solute, PADAC, to assess outer membrane permeability of defensin-treated *E. coli* ML-35p was founded on earlier studies by Zimmerman and Rosselet (21), Nikaido et al. (22), and Bhakdi et al. (23), as described elsewhere (19). Others have used the intact gram-negative outer membrane's ability to exclude small but potentially antimicrobial hydrophobic molecules to assess outer membrane integrity. Among the useful molecular probes in such assays are actinomycin D, Triton X-100, and rifampin (22, 24, 25). Recently, Viljanen et al. assessed the ability of human neutrophil defensins to enhance outer membrane permeability to rifampin or Triton X-100 in selected strains of *E. coli, Salmonella typhimurium,* and *P. aeruginosa* (26). They reported that subinhibitory concentrations of human defensins did not allow ingress of toxic amounts of rifampin or Triton X-100. In contrast, bacteriostatic or bactericidal concentrations of the


A
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defensins did sensitize the organisms to rifampin, suggesting that some degree of defensin-mediated outer membrane permeabilization had occurred. These findings are consistent with the effects of defensins on outer membrane permeability in *E. coli* ML-35p, described in this report.

We suggest that the synchronous inhibition of RNA, DNA, and protein production in defensin-treated bacteria is a consequence of IM permeabilization, with its attendant loss of cellular metabolites and altered ionic cellular environment. This does not preclude a contribution from other effects of defensins yet to be defined.

That normal human PMN kill ingested gram-negative bacteria with considerable efficacy under anaerobic conditions suggests that these cells possess potent O_2-independent bactericidal mechanisms (9, 27, 28). In addition to defensins, the azurophil granules of human PMN contain two other well-characterized components, bactericidal/permeability-increasing protein (B/PI) and cathepsin G, able to kill *E. coli* in vitro. The actions of B/PI have recently been reviewed by Elsbach and Weiss (reviewed in reference 9). It is believed that electrostatic interactions between B/PI, a cationic molecule, and anionic sites in the bacterial outer membrane precede its hydrophobic insertion into the OM, which causes virtually immediate loss of colony-forming activity and increased OM permeability. Although the OM permeability change is reversible, the bactericidal effect is not. Unlike our findings with defensins, viability and general biosynthetic capacity are dissociated in B/PI-treated bacteria, as macromolecular synthesis continues even after viability is lost (9, 24).

Odeberg and Olsson reported that cathepsin G, purified from human PMN, killed *Staphylococcus aureus* 502A and *E. coli* in vitro (4). Interestingly, logarithmic phase *E. coli* that had been exposed to cathepsin G remained viable and synthesized macromolecules normally for ~ 20 min. Thereafter, viability fell and macromolecular synthesis was inhibited in proportion to the decrease in colony count. Although these data are strongly suggestive that cathepsin G may also kill *E. coli* by sequentially attacking OM and IM integrity, this interpretation requires experimental verification.

The interactions between *E. coli* ML-35 and intact human neutrophils were closely examined by Hamers et al. (29), who reported that *E. coli* ML-35 lost its crypticity for β-galactosidase with pseudo-first order kinetics after ingestion by PMN, and that IM perforation (loss of crypticity) and microbial death were correlated. Because our data show that purified defensins can both perforate and kill *E. coli* ML-35 in vitro, defensins may contribute to the ability of intact PMN to kill this organism.

Hamers et al. have also reported that PMN from patients with hereditary myeloperoxidase deficiency or the Chediak-Higashi syndrome showed normal activity against *E. coli* ML-35. Although MPO-derived oxidants such as OCl^- or chloramines may permeabilize the IM of *E. coli* under certain cell-free conditions (30), the observations of Hamers et al. (29) suggest that the MPO-H_2O_2-halide system is not required for this intracellular function of intact human neutrophils. Moreover, because Chediak-Higashi PMN lack cathepsin G and other neutral proteases (31), their findings also suggest that the intraleukocytic permeabilization and killing of *E. coli* ML-35 by normal PMN may not require cathepsin G. Although B/PI is thought to participate in the neutrophil's ability to kill ingested *E. coli* (32), its ability to perforate the IM of susceptible gram-negative organisms in general and to perforate the OM and IM of smooth organisms such as *E. coli* ML-35 (33) is not known.

Almost 20 years ago, Friedberg et al. reported that *E. coli* ML-35 that had been exposed to a crude extract of guinea pig PMN granules lost their crypticity for β-galactosidase and were killed (34, 35). He also reported that treated bacteria developed interesting morphological alterations, including spatial separations between their inner and outer membranes that resemble those shown in Fig. 9. Because at least one defensin (13) is included among the antimicrobial components of guinea pig PMN (36), we suggest that defensins may have contributed to these morphological changes. Rock and Rest (37) recently reported that *N. gonorrhoeae* exposed to extracts prepared from human neutrophil granules developed structural aberrations of the outer membranes with some resemblance to those we noted in defensin-treated *E. coli.* The relationship of these changes to those illustrated in Fig. 10 is presently uncertain.

Our finding that target cell growth and metabolism are required to sensitize *E. coli* ML-35 to the microbicidal effects of human defensins has a counterpart in the earlier studies of Walton and Gladstone (38, 39) and in our more recent studies on the effects of human defensins against *C. albicans*. Walton and Gladstone (38, 39) examined the effects of the abundant, low molecular weight cationic peptides of rabbit PMN on *S. aureus*. Originally discovered by Zeya and Spitznagel (40, 41), who referred to them as lysosomal cationic proteins, these peptides have since been shown to be homologous to human defensins by sequence analysis (12). Walton and Gladstone found that the ability of these rabbit defensins to kill *S. aureus* was highly dependent on the target cell's oxidative metabolism. Staphylococci were substantially protected from these peptides by inhibitors of respiration, energy transfer or oxidative phosphorylation (38, 39). Similarly, *C. albicans* is highly susceptible to human defensins in vitro only when it exhibits active mitochondrial respiration. It is not killed effectively by defensins under anaerobic conditions or in the presence of various mitochondrial inhibitors (15). Although relatively few other examples of the regulation of susceptibility to PMN components by microbial energetics or metabolism have been reported (42, 43), this phenomenon is described with regard to several colicins and bacteriocins (e.g., references 44–46) and serum complement (47).

We have shown that human defensins exert bactericidal activity selectively against metabolically active and growing bacteria, and that they attack the outer and inner membranes of *E. coli* ML-35. It is noteworthy that the ability of defensins to kill fungi (15) and mammalian tumor cells (48) also requires metabolic activity by the target cell. This apparent requirement that target cells participate in their own demise raises

Figure 10. Morphology of defensin-treated *E. coli* ML-35. *E. coli* ML-35 was exposed to HNP-1,2 for 60 min as described in the text. (*A*) A longitudinally sectioned organism with electron-dense material in its periplasmic space (*arrow anchored by the star*) and warty accumulations of electron-dense material adherent to its OM (*arrows*). (*B*) Additional bacteria, sectioned more obliquely, showing numerous discrete electron-dense OM deposits. The warty OM deposits on HNP-treated bacteria did not always show regular spacing.

interesting questions about the general mechanisms of defensin-mediated target cell killing. Perhaps the molecular design of defensins allows them to subvert some component of cellular energetics into a fatal attraction. As defensins can form voltage-regulated ion channels in model membrane systems (49) their broad lethal spectrum may reflect defensin interactions with features of energized membranes, such as the transmembrane potential or proton motive force, that are common to prokaryotic and eukaryotic cells.

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