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The Host Defense Peptide LL-37 Selectively Permeabilizes Apoptotic Leukocytes

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LL-37 is a cationic host defense peptide that is highly expressed during acute inflammation and that kills bacteria by poorly defined mechanisms, resulting in permeabilization of microbial membranes. High concentrations of LL-37 have also been reported to have cytotoxic effects against eukaryotic cells, but the peptide is clearly capable of differentiating between membranes with different compositions (eukaryotic versus bacterial membranes). Eukaryotic cells such as leukocytes change their membrane composition during apoptotic cell death, when they are turned into nonfunctional but structurally intact entities. We tested whether LL-37 exerted specific activity on apoptotic cells and found that the peptide selectively permeabilized the membranes of apoptotic human leukocytes, leaving viable cells unaffected. This activity was seemingly analogous to the direct microbicidal effect of LL-37, in that it was rapid, independent of known surface receptors and/or active cell signaling, and inhibitable by serum components such as high-density lipoprotein. A similar selective permeabilization of apoptotic cells was recorded for both NK cells and neutrophils. In the latter cell type, LL-37 permeabilized both the plasma and granule membranes, resulting in the release of both lactate dehydrogenase and myeloperoxidase. Apoptosis is a way for inflammatory cells to die silently and minimize collateral tissue damage by retaining tissue-damaging and proinflammatory substances within intact membranes. Permeabilization of apoptotic leukocytes by LL-37, accompanied by the leakage of cytoplasmic as well as intragranular molecules, may thus shift the balance between pro- and anti-inflammatory signals and in this way be of importance for the termination of acute inflammation.

The cathelicidins form a large family of microbicidal, cationic host defense peptides found in all mammalian species examined so far, including humans, cows, pigs, rabbits, sheep, mice, monkeys, and horses (60). Only one human cathelicidin, human cationic antimicrobial protein 18 (hCAP-18; unprocessed form), or LL-37 (the predominant, mature antibacterial peptide due to its ability to specifically permeabilize prokaryotic membranes (1, 16, 31); it possesses direct microbicidal activity against bacteria, fungi, and enveloped viruses (5, 33). Very high concentrations of LL-37 have also been reported to have cytotoxic effects against eukaryotic cells in vitro (28). The microbial killing mechanism is dependent on the membrane-active properties of the peptide, which has the ability to interact with both the inner and the outer membranes of gram-negative bacteria (22). The mode of action for the direct microbicidal activity of host defense peptides has not been determined conclusively, but the leading hypothesis is that these peptides are membrane active and have the ability to break the integrity of bacterial cell membranes more or less selectively over eukaryotic membranes (47). Bacterial membranes are composed of phospholipids different from those of eukaryotic cell membranes, with the bacterial membranes being more negatively charged than their eukaryote counterparts. The presence of surface molecules other than phospholipids, e.g., lipopolysaccharide (LPS; an outer membrane component of gram-negative bacteria), is also of importance to the overall negative charge of bacterial membranes. This negative charge is, in part, believed to be a determinant for the interaction with cationic peptides and gives the peptides selectivity for the microbial membranes (37).

Lately, LL-37 has been shown to have immunomodulatory...
properties, in addition to its microbicidal potential (10), and is also capable of activating various immune cells; thus, it possesses a functional dualism that is also displayed by certain other cationic peptides (7, 13, 51, 58). Among the numerous immunomodulatory actions displayed by LL-37, chemotraction and the induction of chemokine release from leukocytes, the stimulation of angiogenesis, and the binding and neutralization of bacterial LPS can be found (9). LL-37 has been shown to be highly upregulated under inflammatory conditions, particularly those involving the skin, e.g., psoriasis (20, 40). During inflammation, it is of vital importance that the activities of the cells involved are tightly regulated. Many inflammatory leukocytes, especially neutrophils, are packed with a variety of cytotoxic substances aimed at microbial killing, and if these substances reach the extracellular environment in an uncontrolled fashion, the risk of tissue destruction and sustained inflammation are increased (25, 56). One important means of ridding the system from potentially harmful intracellular constituents is by apoptosis of the inflammatory cells, followed by phagocytic clearance of the corpse by viable phagocytes (34, 44). Apoptosis is often called the physiological, programmed form of cell death, characterized by the lack of proinflammatory actions and the minimization of damage to surrounding cells and tissue. Apoptotic cells are nonfunctional but, importantly, carry an intact cell membrane that prevents the uncontrolled leakage of intracellular molecules (25). Despite the integrity of the apoptotic cell membrane, its surface-exposed molecule composition differs substantially from that of the membranes of viable cells. One prominent example of surface changes relates to phospholipids. In viable cells, an uneven distribution of phospholipids between the two leaflets of the double-sided membrane is maintained by an energy-dependent process that keeps the negatively charged phosphatidylserine (PS) on the inside. In a nonfunctional apoptotic cell, the PS distribution is leveled out over time, with the PS also being on the outer leaflet as a result (35), making the membrane of apoptotic cells more negatively charged and, in this way, more like the membrane of bacteria. The surface-located PS is also an important “eat-me” flag that ensures phagocytic clearance of the apoptotic cell (18). Annexin V is a Ca\(^{2+}\)-dependent, cell-impermeant protein with a high affinity for PS. When it is conjugated to a fluorochrome, it is widely used for the detection and quantification of apoptosis by flow cytometry.

Given the ability of LL-37 to distinguish between proapoptotic and euapoptotic membranes and the fact that the composition of euapoptotic membranes changes when a cell becomes apoptotic, we investigated whether the peptide could distinguish between viable and apoptotic leukocytes. Using primary human neutrophils, a cell type that has a short half-life in vivo and that rapidly enters apoptosis in culture, we found that LL-37 selectively permeabilized the membranes of apoptotic cells under serum-free conditions, leaving viable cells unaffected in this respect. A specificity similar to that for apoptotic cells was also found for NK cells, another inflammatory leukocyte, which was rendered apoptotic by treatment with hydrogen peroxide. The selective permeabilization of apoptotic neutrophils was very rapid and seemingly independent of known LL-37 surface receptors and/or active cell signaling. Peptide-induced permeabilization in effect shifted apoptotic cells into a necrotic phenotype, whereby intracellular constituents were released into the extracellular milieu; leakage was effectively counteracted by the presence of human serum or high-density lipoprotein (HDL) in a similar manner, as microbicidal killing by LL-37 was blocked by these substances. The mere surface exposure of the negatively charged PS, a consequence of apoptosis, was not responsible for the selective permeabilization of apoptotic cells. We concluded that LL-37 possesses selective permeabilizing activity on apoptotic leukocytes in a manner seemingly analogous to its activity against bacterial membranes. In pathological settings with high levels of cathelicidin expression in the absence of serum constituents (such as psoriatic lesions), the permeabilization of apoptotic leukocytes by LL-37 could be of importance in determining the eventual outcome of acute inflammation.

**MATERIALS AND METHODS**

**Reagents.** LL-37 (LLGDFFRKSKEKKEFKRKEKRFFLRNLVPRTES) was synthesized by N-(9-fluorenylmethoxycarbonyl) chemistry at the Nucleic Acid/Protein Service Unit at the University of British Columbia (Vancouver, British Columbia, Canada). Scrambled LL-37 (LL-37; RSLEGTDFFFPPVRKL NRSKLEFKDKIKQRERKFOVFKL) was purchased from CSS-Albachem Ltd. (East Lothan, United Kingdom). WKYMVM was from Alta Bioscience (Birmingham, United Kingdom), and WRW4 was from GeneScript Corp. (Scotch Plains, NJ). Hp(2-20) was synthesized and purified by high-pressure liquid chromatography (Innovagen, Lund, Sweden). Ficol-Paque was from Pharmacia (Uppsala, Sweden), and Ficol-Hypaque was from Nycomed (Oslo, Norway). Dextran was purchased from Amersham Biosciences (Uppsala, Sweden). The A-6013 agarose used for the casting of all plates for the inhibition zone assays was from Sigma Chemical Co. (St. Louis, MO), as were oxidized ATP (oxATP), HDL, cytochalasin B, lidocaine, colchicine, and calcine. The antibody α-CD95 was from NordicBioSite (Täby, Sweden), Annexin V-fluorescein isothiocyanate (FITC), annexin V-allophycocyanin (APC), 7-amino-actinomycin D (7-AAD), and the BD IMag NK cell isolation kit were supplied by BD Biosciences (San Jose, CA). RPMI 1640 medium was from PAA Laboratories (Pasching, Austria). The lipids used for liposome preparation (1,2-dipalmitoyl-sn-glycerol-3-phosphatidylethanolamine [DPPPE], 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylethanolamine [DPPPC], 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylserine [DOPPS], and cholesterol) were purchased from Larodan (Malmö, Sweden).

**Isolation and culture of human cells.** Human neutrophils were isolated by a standard technique (11, 12) from buffy coats obtained from the blood of healthy donors at the Sahlgrenska Hospital, Gothenburg, Sweden. In short, dextran sedimentation was performed at 1 × g, followed by hypotonic lysis of the remaining erythrocytes and centrifugation in a Ficoll-Paque gradient. Thereafter, the neutrophils were washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum to a density of 5 × 10^6 cells/ml. Cells (500 μl) were added to polypropylene tubes and incubated at 37°C in 5% CO\(_2\) for 6 h at 37°C (23, 24, 53). The selective permeabilization of apoptotic cells into a necrotic phenotype, whereby intracellular constituents were released into the extracellular milieu; leakage was effectively counteracted by the presence of human serum or high-density lipoprotein (HDL) in a similar manner, as microbicidal killing by LL-37 was blocked by these substances. The mere surface exposure of the negatively charged PS, a consequence of apoptosis, was not responsible for the selective permeabilization of apoptotic cells. We concluded that LL-37 possesses selective permeabilizing activity on apoptotic leukocytes in a manner seemingly analogous to its activity against bacterial membranes. In pathological settings with high levels of cathelicidin expression in the absence of serum constituents (such as psoriatic lesions), the permeabilization of apoptotic leukocytes by LL-37 could be of importance in determining the eventual outcome of acute inflammation.
Evaluation of apoptosis. The neutrophils that had been incubated overnight, as described above, were washed and resuspended in annexin V binding buffer (AVBB; 1 mM HEPES, 14 mM NaCl, 0.25 mM CaCl2; pH 7.4) to 6.25×10⁸ cells/ml. Samples (70 µl) were stained with 5 µl annexin V-FITC for 5 min at ambient temperature, whereupon 7-AAD was added, and the staining was continued for 10 min before the addition of AVBB to give a final volume of 500 µl. In some experiments, annexin V-FITC was exchanged for a CD16-FITC. 100-µl samples were stained with 10 µl antibody for 10 min at 0°C, whereupon staining with 7-AAD was performed as described above. Peptides diluted in water were added 5 min prior to analysis, if not indicated otherwise. HDL (650 µM), bovine serum albumin (0.5%; w/vol), or 10% salt mimicking serum salt (11.3 mM NaCl, 2.4 mM NaHCO3, 0.06 mM MgCl2, 0.13 mM CaCl2, 0.39 mM KCl) (28) was added to the indicated samples before the addition of peptide. Samples were analyzed by fluorescent-activated cell sorter analysis before and after the addition of peptides. For samples treated with the formyl peptide receptor-like 1 (FPRL1) antagonist, WW4 (5 µM) was added 5 min (at 37°C) prior to the addition of peptide. The same procedure was used for the P2X7 inhibitor oxATP (900 µM), but by the procedure with oxATP, the samples were preincubated with the inhibitor for 30 min. The concentrations and incubation times for the inhibitors have previously been shown to specifically block Ca²⁺ flux induced through FPRL1 and P2X7, respectively (15). The inhibitors of membrane repair cytotoxin-like (1) (FPRL1) antagonist, WW4 (5 µM), or lidocaine (5 mM) were coincubated with the cells overnight and were added to all buffers used for the subsequent experiments, as indicated.

The H₂O₂-treated NK cells were washed and resuspended in AVBB. Each sample was stained at ambient temperature with 5 µl annexin V-FITC for 5 min, followed by staining with 5 µl 7-AAD, for a final volume of 100 µl, for another 10 min. After the staining, the mixture was then centrifuged at 4000 g at 4°C for 10 min with AVBB before the analysis. Flow cytometry was performed before and 5 min after the addition of LL-37 (5 µg/ml) was used for NK cells, as these cells are more susceptible than neutrophils to the cytotoxic action of LL-37.

Determination of direct microbicidal activity. Escherichia coli strain MG1655 was grown overnight in Luria-Bertani (LB) broth (3) at 37°C on a rotary shaker. A modified inhibition zone assay was used for the detection of direct microbicidal activity (21). In short, standard LB agar (LB broth supplemented with 1% [w/vol] agarose) containing bacteria (approximately 5×10⁸ CFU in logarithmic growth phase per milliliter agar) was poured into petri dishes (diameter, 92 mm). Wells (diameter, 3 mm; depth, 1 mm) were punched in the agar, and peptide preparations (3 µl) diluted in distilled water were added to the wells, whereupon the plates were incubated at ambient temperature for 45 min and then at 37°C overnight. The peptide was used in the assays at amounts ranging from 1.88 µg/ml to 7.5 µg/ml. Inhibition zones were calculated as the radius of the clear zones, free of visible bacteria, surrounding the wells. HDL (650 µg/ml) was added to the agar plates before the plates were cast.

Liposome preparation. Lipids (DPPE, DPPC, DPPG, and cholesterol) were dissolved in chloroform-methanol (1:1) to a concentration of 20 mg/ml. DOPS was dissolved in chloroform to 25 mg/ml. The lipid mixtures (final concentration of total lipids, 2.5 mM) were dissolved in chloroform-methanol (1:1) and dried at reduced nitrogen pressure at 45°C, with subsequent drying under vacuum for 1 h. Liposomes containing the following were prepared: DPPE-DPPC-cholesterol (1:1:1; lipids typically found in eukaryotic membranes) and DPPE-DPPG (7:3; phospholipids typically found in bacterial membranes), and DPPE-DPPC-cholesterol-DOPE (3:3:1:1; lipids typically found in eukaryotic plasma membranes with 10% PS). During the subsequent preparation steps, the liposomes were kept at 50°C at all times to avoid solidification of the dipalmityl lipids. The dried lipid film was dissolved in 1 ml Tris buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA; pH 7.4) containing self-quenching concentrations of calcein (80 µM) and DCFDA (25 µM) was added 5 min (at 37°C) prior to the addition of peptide. The same procedure was used for the P2X7 inhibitor oxATP (900 µM), but by the procedure with oxATP, the samples were preincubated with the inhibitor for 30 min. The concentrations and incubation times for the inhibitors have previously been shown to specifically block Ca²⁺ flux induced through FPRL1 and P2X7, respectively (15). The inhibitors of membrane repair cytotoxin-like (1) (FPRL1) antagonist, WW4 (5 µM), or lidocaine (5 mM) were coincubated with the cells overnight and were added to all buffers used for the subsequent experiments, as indicated.

RESULTS

Direct microbicidal action of LL-37 and discrimination between membranes of different compositions. LL-37 is a host defense peptide with dual actions that both works directly by permeabilizing microbial membranes and has receptor-dependent, immunomodulatory actions on leukocytes as a means of protecting against infections. The direct microbicidal effects of LL-37 and the sLL-37 control peptide against E. coli were assessed in inhibition zone assays; LL-37 induced a clear zone around the well that was free of visible bacteria, while sLL-37 had no direct microbicidal effect (Fig. 1A). The lethal concentration was calculated to 6.3 µg/ml for E. coli (strain MG1655) under the specific medium conditions used in the assay. The lethal concentration was determined from the diameter of the inhibition zone by a previously described method (26) and was defined as the lowest concentration that just inhibited colony formation in the thin agar plates. Under the serum-free conditions used, LL-37 (100 µg/ml) had no effect on the integrity of the cell membranes of freshly prepared neutrophils, as shown by a lack of 7-AAD staining (Fig. 1B). This dye is incapable of passing intact cell membranes and therefore functions as a useful marker of membrane integrity (46). These experiments show that LL-37 can be directly microbicidal in the 5-µg/ml range (as determined by calculation of the lethal
concentration from the results obtained from the inhibition zone assays), whereas concentrations up to 100 μg/ml were not cytotoxic for neutrophils under serum-free conditions.

To further characterize the ability of LL-37 to discriminate between bacterial and eukaryotic membranes, we tested the effect of LL-37 on liposomes containing the lipids typically found in bacterial as well as in eukaryotic membranes in a calcein release assay. Bacterial membranes are slightly more negatively charged than eukaryotic membranes, and liposomes containing large amounts of neutral PE and the negatively charged phosphatidylglycerol (PG) were synthesized. For the eukaryotic membrane-like liposomes, cholesterol was incorporated together with the two neutral phospholipids PE and PC.

As expected, LL-37 readily permeabilized the bacterial membrane-like liposomes (Fig. 1C). The eukaryotic membrane-like liposomes were permeabilized to a limited extent (approximately 15%), but they were obviously much more resistant than the bacterial membrane-like liposomes to the lytic action of LL-37. Taken together, these data show that LL-37 is clearly capable of differentiating between membranes with different compositions.

Selective permeabilization of apoptotic neutrophils by LL-37. LL-37 had no cytotoxic effect on freshly prepared human neutrophils (Fig. 1B). In order to study the effect of LL-37 on viable and apoptotic membranes of primary leukocytes, we employed human neutrophils, which gradually enter spontaneous apoptosis during in vitro culture. In this set of experiments, neutrophils incubated overnight spontaneously entered apoptosis, leaving a mixed population of cells with 66% viable cells (annexin V negative, 7-AAD negative) and 31% apoptotic cells (annexin V positive, 7-AAD negative), as assessed by flow cytometry (Fig. 2A, left panel). By use of this standardized protocol, very few cells (less than 3%) displayed a necrotic phenotype with permeable membranes, as indicated by the lack of 7-AAD-positive events. The use of this mixed culture enabled us to investigate viable and apoptotic cells simultaneously, and addition of LL-37 (50 μg/ml, 5 min of incubation) to the mixed population selectively permeabilized the apoptotic cells that acquired the 7-AAD stain under serum-free conditions (Fig. 2A, middle panel, upper right quadrant). The membranes of annexin V-negative cells were still intact (7-AAD negative) after the addition of peptide (Fig. 2A; middle panel, lower left quadrant). sLL-37 (50 μg/ml, 5 min of incubation) did not permeabilize any cell type (Fig. 2A, right panel), indicating that the cationic nature of LL-37 is not sufficient to permeabilize apoptotic neutrophils. sLL-37 at a

FIG. 1. LL-37 can distinguish between membranes with different compositions. (A) The direct microbicidal activity of LL-37 (1.88, 3.75, and 7.5 μg/well) against E. coli was determined in an inhibition zone assay. LL-37 gave rise to clear, bacteria-free zones around the wells (upper panel), while sLL-37 (1.88, 3.75, and 7.5 μg/well) was inactive (lower panel). (B) Freshly separated neutrophils were not sensitive to the direct microbicidal, lytic action of LL-37 (100 μg/ml), as shown by the lack of 7-AAD fluorescence intensity plotted against the forward scatter. 7-AAD is a nucleic acid stain used for the detection of the loss of membrane integrity in cells. The results of one experiment with the percentage of cells in each region are shown. (C) LL-37 (5 ng/ml) readily permeabilized bacterial membrane-like liposomes (gray line) composed of PE-PG (7:3), while the eukaryotic membrane-like liposomes (black line), composed of PE-PC-cholesterol (1:1:1), were more resistant to the permeabilizing effect of LL-37. The percentage of total calcein released, as assessed by the addition of Triton X-100, is plotted against time. The means ± SDs (n = 3) obtained at 0, 50, 100, 150, and 200 s are shown. The amounts of calcein released at 100, 150, and 200 s were compared; and significant differences between the amounts released were established (P < 0.01 at each point, as indicated [**]; n = 3). The arrow indicates the time of addition of peptide.
concentration of 500 \( \mu \)g/ml was also ineffective (data not shown).

In order to rule out the possibility that an undefined interaction between LL-37 and annexin V caused the permeabilization, experiments were conducted with CD16 instead of annexin V as a marker for neutrophil apoptosis (Fig. 2B, left panel). CD16 is a surface protein present on viable neutrophils (Fig. 2B, left panel, lower right quadrant) that is lost upon apoptosis (Fig. 2B, left panel, lower left quadrant), and only cells negative for CD16 (i.e., apoptotic cells) became 7-AAD positive (Fig. 2B, right panel). After addition of LL-37, the apoptotic cell population was permeant not only to the nuclear dye 7-AAD but also, apparently, to the \( \alpha \)-CD16 antibody that gained access to intracellular CD16 and, as a result, conferred a CD16 signal to the permeabilized cells (Fig. 2B, right panel).

**Selective permeabilization of apoptotic neutrophils by LL-37.** Inflammatory cells other than neutrophils have the ability to enter apoptosis as well. NK cells are cytotoxic, large granular lymphocytes important for eradicating viruses and combating tumor cells. One efficient way to drive NK cell apoptosis is to subject these cells to extracellular reactive oxygen species (23). To test whether the selective permeabilization of apoptotic cells applied only to neutrophils or if it represents a more general mechanism that acts on other leukocytes as well, NK cells were isolated and subjected to \( \mathrm{H}_2\mathrm{O}_2 \) treatment for the induction of apoptosis. Untreated NK cells incubated for 6 h displayed only 5% apoptotic cells, as assessed by annexin V and 7-AAD labeling (Fig. 3A). When the cells were coincubated with \( \mathrm{H}_2\mathrm{O}_2 \) (200 \( \mu \)M), a larger population (17%) became apoptotic (Fig. 3B), resulting in a mixed population of viable and apoptotic NK cells. A population of NK cells (16%) also appeared to be permeabilized after the addition of \( \mathrm{H}_2\mathrm{O}_2 \) (Fig. 3B, upper right quadrant). After this mixed population was washed, it was treated with LL-37 (5 \( \mu \)g/ml, 5 min of incubation), and with these leukocytes, the apoptotic cells were also

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**FIG. 2.** Selective permeabilization of apoptotic neutrophils by LL-37. (A) Neutrophils incubated for 20 h spontaneously entered apoptosis, resulting in 66% viable cells and 31% apoptotic cells, as assessed by annexin V and 7-AAD staining and visualized by flow cytometry (left panel). When LL-37 (50 \( \mu \)g/ml) was added to these cells, all apoptotic (annexin V-positive) cells were permeabilized (7-AAD positive), while the viable (annexin V-negative) population was unaffected (middle panel) 5 min after addition of the peptide. sLL-37 (50 \( \mu \)g/ml) was inactive when it was added to the system (right panel). The results presented here represent those of one of three experiments performed for this experimental series. (B) Apoptosis was also assessed by CD16 shedding (left panel). Only the apoptotic neutrophils (CD16 negative) were permeabilized by the addition of LL-37 and stained positive for 7-AAD. The \( \alpha \)-CD16 antibody also gained access to intracellular CD16 upon permeabilization, as shown by the increase in the fluorescence intensity of CD16 (right panel). The plots represent those from one of two independent experiments that were performed. Each plot shows the means ± SDs from one representative experiment.
cells also increased upon addition of H$_2$O$_2$ (from 5.3 $\times$ 10$^3$ to 5.6 $\times$ 10$^3$ viable cells) compared to those for the H$_2$O$_2$-treated control cells, no significant difference was found ($P = 0.07; n = 3$). A lower standard concentration of LL-37 was used against the NK cells than against the neutrophils (5 $\mu$g/ml and 50 $\mu$g/ml, respectively), owing to the fact that NK cells were more susceptible to the cytotoxic action of LL-37. At 10 $\mu$g/ml, a substantial amount of cytotoxicity against NK cells was observed (the proportion of viable cells dropped from 82.6% to 38.5%; data not shown). At 5 $\mu$g/ml of LL-37, mainly apoptotic (annexin V-positive) NK cells were permeabilized, whereas the permeabilization of viable cells was not statistically significantly reduced. These data indicate that the selective permeabilization of apoptotic cells is independent of the type of leukocyte.

Characterization of selective permeabilization. Dose titration experiments revealed that the permeabilizing effect on apoptotic neutrophils was concentration dependent starting at approximately 10 $\mu$g/ml, and a full effect was reached at 50 $\mu$g/ml of LL-37. The half-maximal effective concentration (EC$_{50}$) was determined for the selective permeabilization of apoptotic cells by plotting the mean value of the percentage of apoptotic cells at concentrations 0, 5, 10, and 50 $\mu$g/ml for three separate experiments and fitting a straight line to the data set ($R^2 = 0.89$); the EC$_{50}$ for the permeabilization of apoptotic neutrophils was 27 $\mu$g/ml. At 500 $\mu$g/ml, a more general cytotoxic effect was observed; in addition to the permeabilization of all apoptotic cells, viable neutrophils were also affected (Fig. 4A) ($P < 0.001$ compared to the results for viable neutrophils treated with buffer). The EC$_{50}$ was also determined for the cytotoxic action of LL-37 against viable cells by plotting the mean value of the percentage of viable cells obtained after treatment with LL-37 at concentrations of 0, 5, 10, 50, 100, and 500 $\mu$g/ml from three separate experiments. A straight line was fitted to the data set ($R^2 = 0.97$), the trend line was extrapolated, and the EC$_{50}$ was determined to be 700 $\mu$g/ml. Due to technical limitations, we could not use concentrations of LL-37 higher than 500 $\mu$g/ml. On the basis of EC$_{50}$, the concentration needed for the permeabilization of viable neutrophils (general cytotoxicity) was found to be approximately 25 times higher than that needed for the permeabilization of apoptotic neutrophils. Time and titration experiments showed that the specificity was intact (i.e., viable cells were unaffected) for at least the first 5 min ($P > 0.05$ compared to the results obtained by treatment with buffer alone). Almost all annexin V-positive cells became leaky within 5 min of LL-37 addition (Fig. 4B). In an attempt to study the leakage within the first 5 min, we analyzed the process (7-AAD fluorescence) kinetically in a flow cytometer, having first gated the cells on the basis of annexin V staining (Fig. 4C, left panel). Increased 7-AAD staining was apparent immediately after addition of LL-37 (Fig. 4C, right panel) in the annexin V-positive cells (red), whereas the annexin V-negative population (green) never became 7-AAD positive. These results again showed that only apoptotic cells were permeabilized by LL-37 and that the leakage started immediately after addition of peptide.

Cationicity alone was not sufficient to confer direct microbicidal action (Fig. 1A) or specific permeabilization (Fig. 2A, right panel), since sLL-37 displayed no activity in these assays (at the highest concentrations used of 7.5 $\mu$g/well and 500 $\mu$g/ml, respectively). We next tested the direct microbicidal and immunomodulatory peptide Hp(2-20), which combines cationicity with an alpha-helical nature, i.e., classical features of cationic host defense peptides (13). This peptide was unable to permeabilize leukocytes (viable or apoptotic) up to concentrations of 2 mM (data not shown), showing that the selective action described for LL-37 is not a general feature of cationic, alpha-helical host defense peptides.

The effect of LL-37 on apoptotic neutrophils is independent of known surface receptors and Ca$^{2+}$ signaling. LL-37 has been reported to employ a variety of surface receptors for the mediation of its immunomodulatory effects; for neutrophils, two of the most likely receptor candidates are FPRL1 and P2X$_7$ (17, 39). We used the antagonists WRW4 (4) and oxATP (38) to block FPRL1 and P2X$_7$, respectively. In the presence of these two antagonists, the apoptotic cells were still permeab-
likely independent of surface receptors and/or active cell signaling. In this respect, the permeabilizing effect was different from other (mostly receptor-dependent) immunomodulatory effects on leukocytes reported previously (10). In conclusion, the effect of LL-37 on apoptotic leukocytes is closely related to the direct microbicidal, permeabilizing effect of LL-37 rather than immunomodulating, receptor-dependent effects.

**Inhibition by serum components.** We hypothesized that the mechanism behind the observed specific permeabilization of apoptotic cells by LL-37 had more in common with its direct microbicidal effects than its immunomodulatory actions. The direct microbicidal effect of LL-37 is known to be inhibited by various serum components like HDL and apolipoprotein A1 (32, 55), and in our microbicidal assay, the presence of HDL also markedly inhibited bacterial killing by the peptide. When HDL was added to the inhibition zone plates at levels expected to be found in serum (650 μg/ml), the inhibition zone size generated by LL-37 was markedly decreased (Fig. 6A).

We next subjected a mixed population of neutrophils (viable and apoptotic) to LL-37 in the presence or the absence of either 10% human serum or HDL (650 μg/ml). Both serum and HDL completely blocked the LL-37-induced permeabilization of apoptotic neutrophils (Fig. 6B). The immunomodulatory, receptor-dependent effects of LL-37 have previously been shown to be intact in the presence of serum (17), in which HDL is a prominent protein. These data further strengthen the hypothesis that the observed effect is, in fact, receptor independent. When a salt solution composed to mimic the composition of 10% serum or bovine serum albumin (0.5%; wt/vol) was added, no inhibitory effects were observed (data not shown). These data imply that the presence of proteins in general and serum salts does not impair the function of LL-37.

**Negatively charged PS is insufficient to mediate membrane permeabilization by LL-37.** One plausible cause of the specific action of LL-37 on apoptotic cells could be the changes in membrane composition that accompany apoptosis, such as the surface exposure of the phospholipid PS. This negatively charged molecule could theoretically confer an affinity for the positively charged peptide, similar to the reported electrostatic interaction between LL-37 and LPS (30, 31). We checked whether LL-37 displays a direct affinity for PS by testing whether it binds to phospholipids and glycosphingolipids immobilized on a solid phase (50). When an attempt was made to bind LL-37 to total lipid extracts from human erythrocytes (40 μg) and control mixtures of acid glycosphingolipids from various sources (40 μg each), which were separated on thin-layer chromatograms, or to pure PE, PC, and PS (4 μg each), no specific binding of LL-37 to any of these compounds was observed (data not shown). In a microtiter well assay, the anionic GM1 ganglioside [Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer] and the neutral phospholipid PE were included as controls. LL-37 exerted no specificity toward PS compared to its specificity toward the other two lipids found in eukaryotic membranes included in this assay (data not shown).

We next checked whether the presence of PS could contribute to making membranes sensitive to the lytic action of LL-37 by incorporating PS into liposomes containing the phospholipids typically found in eukaryotic membranes and subsequently measuring the level of calcein leakage after addition of the peptide. The incorporation of PS into liposomes containing...
phospholipids typically found in eukaryotic membranes did not confer any permeabilizing activity on LL-37 (Fig. 7). The total amounts of calcein released (determined by calculation of the area under the curve) for the two liposome preparations were determined and when the means were compared, no significant difference between the two was found ($P = 0.08; n = 3$).

The presence of PS did not change the susceptibilities of the liposomes to the lytic activity of LL-37, and the peptide did not seem to have any direct affinity for PS. We next hypothesized that LL-37 does not have any specific affinity for the apoptotic cells but instead interacts with both viable and apoptotic cells to the same extent and in this way induces limited membrane damage. The differences in the outcomes would then be due to the fact that the nonfunctional apoptotic cells are unable to repair the disturbed membrane, whereas the viable cells are quick to reseal the disruption. Viable cells are equipped with a complex machinery that allows them to repair membrane damage, and that machinery often involves a variety of cytoskeletal...

![FIG. 5](image_url)

**FIG. 5.** The effect of LL-37 on neutrophils is independent of known receptors and Ca$^{2+}$ signaling. (A) A mixed population of viable and apoptotic neutrophils was treated with the two antagonists WRW4 (5 μM) and oxATP (900 μM), which antagonize two known LL-37 neutrophil receptors, FPRL1 and P2X₇, respectively. In the presence of the antagonists, LL-37 (50 μg/ml) still permeabilized apoptotic, annexin V-positive cells. Paired t tests were performed to establish statistical significance ($n = 3$). (B) An overnight preparation of incubated neutrophils was assessed for annexin V staining, and a mixed population of both viable (green) and apoptotic (red) neutrophils (left panel) was shown. The mixed population was stimulated with the potent neutrophil FPRL1 ligand WKYMVM (10⁻⁷ M), and the transient Ca$^{2+}$ release was monitored by flow cytometry. The viable cells (green line) responded with a rise in the intracellular Ca$^{2+}$ concentration, while the apoptotic cells (red line) were totally irresponsive. The results of one representative experiment are shown. The FL-1/FL-3 ratio was plotted against time (right panel). *, $P < 0.05$; ***, $P < 0.001$.

![FIG. 6](image_url)

**FIG. 6.** The permeabilizing effect of LL-37 on apoptotic neutrophils is inhibited by human serum and HDL. (A) When HDL (650 μg/ml) was added to the inhibition zone plates, the clear zone surrounding the well after the addition of LL-37 (1.88 μg/well) decreased markedly. (B) In a control experiment, apoptotic neutrophils were readily permeabilized by the addition of LL-37 (50 μg/ml; left panel). When LL-37 was added to cells in a solution containing 10% normal human serum, the permeabilizing effect was totally blocked (middle panel). The addition of HDL (650 μg/ml) to the neutrophil population prior to the addition of LL-37 (50 μg/ml) also inhibited the permeabilizing effect of LL-37 on apoptotic neutrophils (right panel). Representative plots from three independent experiments and means ± SDs summarized from three independent experiments are shown.
filaments such as actin and microtubuli (36). We therefore subjected mixed neutrophil populations containing both viable and apoptotic cells to different inhibitors of cytoskeletal filament function in order to see whether such treatment would also enable the permeabilization of viable cells. Treatment of cells with cytochalasin B (which inhibits actin polymerization) (29) or colchicine (which perturbs microtubule assembly) (8) before addition of LL-37 did not make the viable cells sensitive to the lytic action of LL-37 (data not shown). In addition, the local anesthetic lidocaine, which is known to modify cell membrane fluidity (59), did not alter the pattern of susceptibility to LL-37; i.e., the peptide still permeabilized only the annexin V-positive cells (data not shown).

Permeabilization of apoptotic cells by LL-37 is associated with leakage of intracellular as well as intragranular constituents. Among the inflammatory leukocytes, neutrophils harbor an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an intact cell membrane retain their intracellular substances, and released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsenal of proteolytic enzymes that could be very harmful to surrounding cells and tissues if they were released in an uncontrolled fashion. Apoptotic cells with an especially potent arsen
permeabilization of apoptotic cells was seen for NK cells rendered apoptotic by hydrogen peroxide treatment, indicating that the effect was not limited to neutrophils. NK cells were more susceptible to the lytic action of LL-37, and at concentrations higher than 5 μg/ml, LL-37 displayed a more general cytotoxicity against purified NK cells. Viable neutrophils were not permeabilized until LL-37 concentrations exceeded 100 μg/ml, and it makes sense that neutrophils, being important reservoirs for LL-37, should be more resistant to peptide-induced membrane disruption.

The immunomodulatory actions of LL-37, as opposed to its direct microbicidal effects, are primarily mediated by receptor binding. The surface receptors most often implicated in LL-37-mediated responses in neutrophils are the chemoattractant receptor FPRL1 (17) and the nucleotide receptor P2X7 (39). Antagonists for these receptors, WRW4 (an antagonist of FPRL1 [4] and oxATP (an antagonist of P2X7 [38]), did not affect the LL-37-induced permeabilization of apoptotic neutrophils, nor did they influence the susceptibilities of viable cells. These findings suggest that neither FPRL1 nor P2X7 is involved in the specific permeabilization of apoptotic cells. We also showed that the apoptotic cells in our mixed neutrophil samples were completely unable to mobilize intracellular calcium in response to FPRL1 activation, indicating that these cells are, in fact, nonfunctional and incapable of transmitting intracellular signals. Taken together, the permeabilization of apoptotic cells is highly unlikely to depend on the interaction of LL-37 with surface receptors or even active signal transduction. This makes the effect of LL-37 on apoptotic leukocytes appear to be much more like its direct microbicidal action than its immunomodulatory functions. The fact that the presence of blood constituents (serum or HDL) that inhibit LL-37’s direct microbicidal effect was also effective at blocking the permeabilization of apoptotic cells supports this view.

LL-37 has previously been shown to inhibit apoptosis (39) as well as promote the necrosis (6) of human neutrophils, and it is likely that the induction of secondary necrosis could explain the former finding, as was proposed in a study published during the preparation of the manuscript (61). In line with this, our data presented here imply that in the presence of LL-37, cells that spontaneously enter apoptosis are rapidly permeabilized and are eventually lost from subsequent analyses. The viable cells, however, would be unaffected by the peptide, resulting in an apparent enrichment of viable cells at the expense of apoptotic cells, which could lead to the false impression that LL-37 inhibits apoptosis. We also show that the LL-37-induced permeabilization of apoptotic cells destroys the integrity of the plasma membrane, as seen by the leakage of the cytoplasmic enzyme LDH to the extracellular space. In addition, we could also see the leakage of MPO, which is stored in the azurophil granules (19). Since this granule type is responsible for storing most of the proteolytic enzymes (19) as well as the immunomodulatory molecules, this finding is important and indicates that LL-37-mediated permeabilization could be a potent means of getting such substances out to an extracellular location. The functional consequences of releasing tissue-destroying enzymes in combination with molecules that facilitate wound healing remain to be determined. Interestingly, the levels of MPO released were lower than anticipated, on the basis of the proportion of apoptotic cells; it is possible that LL-37 interacts electrostatically with anionic membrane components and sticks to the plasma membrane during permeabilization. The permeabilization of azurophil granules could also require a time longer than the time required for permeabilization of the plasma membrane. One must also take into account the possible release of hCAP-18 from the specific granules in neutrophils upon permeabilization. If LL-37 is cleaved extracellularly by protease 3, the accumulation of additional LL-37 could result in a positive-feedback loop.

The mere cationicity of a peptide is not sufficient to induce specific permeabilization, since sLL-37 was inert in terms of direct microbicidal activity as well as in the permeabilization of apoptotic cells. Cationicity combined with an alpha-helical structure was also not enough to permeabilize apoptotic cells, since Hp(2-20) did not possess this ability, even though it potently kills bacteria (43). This Helicobacter pylori peptide is in many ways similar to LL-37 and also shares specificity for FPRL1 as a neutrophil receptor (13). We first hypothesized that flipping of the negatively charged PS from the inner leaflet of the lipid bilayer to the outer leaflet could confer susceptibility to the cationic peptide LL-37. Several studies have shown that cationic peptides have an increased affinity (and killing capacity) for cancer cells compared to their affinity for healthy cells and that this is due to the fact that cancer cells have more negatively charged plasma membranes (54, 62). This charge difference partly originates from the various amounts of PS present on the outer leaflet of the plasma membranes (41). However, our data generated from liposome studies and chromatogram binding assays failed to support a direct role for PS. It is likely that apoptotic membrane changes other than the flipping of PS could explain the selective action of LL-37 on the apoptotic membranes.

In striking contrast to most immunomodulatory functions of LL-37 that are intact or that even depend on the presence of serum (48), we found that serum completely blocked the permeabilization of apoptotic cells. In this respect, the effect that we describe seems to be much more related to the direct microbicidal action of LL-37. With this in mind, it is reasonable to assume that the ability of LL-37 to permeabilize apoptotic cells in vivo would be of importance mainly at sites devoid of serum components, such as the skin or the lungs. LL-37 is very highly expressed in psoriatic scales of the skin and contributes to keeping the scales free of microbial pathogens (40). In addition, psoriatic scales are characterized by the continued presence of neutrophils, indicative of a chronic state of acute inflammation (52). Our findings that LL-37 induces permeabilization of apoptotic leukocytes, concomitant with the leakage of both cytoplasmic and granular proteins, could thus help explain why a state of acute inflammation fails to resolve at sites rich in LL-37 and deprived of serum components.

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