



Published in final edited form as:

*Curr Eye Res.* 2005 May ; 30(5): 385–394.

## Human Cathelicidin (LL-37), a Multifunctional Peptide, is Expressed by Ocular Surface Epithelia and has Potent Antibacterial and Antiviral Activity

**Y. Jerold Gordon**

*The Charles T. Campbell Ophthalmic Microbiology Laboratory, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

**Ling C. Huang**

*University of Houston, College of Optometry, Houston, Texas, USA*

**Eric G. Romanowski and Kathleen A. Yates**

*The Charles T. Campbell Ophthalmic Microbiology Laboratory, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

**Rita J. Proske and Alison M. McDermott**

*University of Houston, College of Optometry, Houston, Texas, USA*

### Abstract

*Purpose:* This study determined whether LL-37 (cathelicidin) is expressed by conjunctival and corneal epithelia as part of ocular host defense. The antimicrobial activity of LL-37 was also assessed *in vitro* against *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE), herpes simplex virus type 1 (HSV-1), and adenovirus (Ad). *Methods:* Expression of LL-37/hCAP 18 mRNA and LL-37 protein was determined by reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting, respectively, in scraped human corneal epithelium and primary cultured human corneal and conjunctival epithelial cells. The EC<sub>50</sub> values for three strains of PA and one each of SA and SE were determined for LL-37. LL-37 antiviral inhibition of HSV-1 and adenovirus was assessed by direct inactivation assays. Toxicity of LL-37 to A549 cells was evaluated by a MTT assay. *Results:* LL-37/hCAP18 mRNA and LL-37 peptide were expressed by human corneal and conjunctival epithelial cells. Antibacterial activity for LL-37 was demonstrated (EC<sub>50</sub> values for the three PA strains were 2.8 ± 1.3, 1.9 ± 0.3, and 3.6 ± 2.1; for SA: 1.6 ± 1.5; for SE: 1.3 ± 1.9 µg/ml). LL-37 produced a significant reduction (p < 0.001 ANOVA) in HSV-1 and Ad19 viral titers with distinctly different time-kill curves (p < 0.001). LL-37 (up to 111 µM) produced no toxicity in A549 cells. *Conclusions:* Corneal and conjunctival epithelia express LL-37 as part of mucosal innate immunity to protect against bacterial and viral ocular infections.

### Keywords

adenovirus; antimicrobial peptides; cathelicidin; HSV-1; LL-37; pseudomonas

### INTRODUCTION

The innate immune system is an ancient, complex, rapid killing, nonspecific, redundant multifunctional system that is highly effective in protecting the host.<sup>1</sup> Scientific interest in this

field is burgeoning as reflected by the growing number of publications including several excellent reviews.<sup>2-5</sup> Studies over the past two decades have shown that small cationic peptides with potent antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and some viruses are an important component of the innate immune system.<sup>6-9</sup> Initially described in leukocytes, cationic antimicrobial peptides such as defensins and cathelicidins are also secreted by various epithelial tissues.<sup>2,10</sup> The precise mechanism of antimicrobial activity of these peptides is incompletely understood, but it is widely accepted that perturbation of the microbial cell membrane is the major means by which these peptides kill.<sup>11,12</sup> In addition to their antimicrobial activity, additional diverse functions for these peptides include important roles in inflammation, immune activation, and wound healing.<sup>13-15</sup>

Cathelicidins consist of a highly conserved N-terminal region (the cathelin domain) with homology to the cysteine protease inhibitor cathelin and a less conserved C-terminal antimicrobial region that varies among species, yielding multiple peptides with a remarkable variety of sizes, sequences, and structures.<sup>16</sup> Only one cathelicidin has been described in humans, which is human cationic antimicrobial peptide of 18 kDa (hCAP18). This peptide is expressed by neutrophils and epithelial tissues lining the oral cavity and respiratory, urogenital, and gastrointestinal tracts.<sup>17-19</sup>

Previous studies have shown that hCAP18 is upregulated by inflammation and injury.<sup>20-24</sup> hCAP18 is cleaved by the action of serine proteases to generate the 37-amino-acid antimicrobial peptide LL-37 and a 103-amino-acid cathelin domain.<sup>25</sup> A recombinant version of the hCAP18 cathelin domain was also recently shown to have antimicrobial activity and to inhibit protease activity.<sup>26</sup> LL-37 has been shown to have potent antimicrobial activity against a variety of bacteria including staphylococcal species and *Escherichia coli* and fungi such as *Candida albicans*.<sup>20,27,28</sup> The antiviral activity of LL-37 has received far less attention, but some activity has been reported against herpes simplex virus (HSV)<sup>29</sup> and vaccinia virus.<sup>30</sup>

In addition to its antimicrobial function, LL-37 is chemotactic for monocytes, T cells, neutrophils, and mast cells, stimulates mast cell histamine release, modulates dendritic cell differentiation, and stimulates IL-8 secretion.<sup>14,31-34</sup> LL-37 has also been shown to stimulate angiogenesis,<sup>35</sup> and lack of LL-37 activity was associated with impaired epithelial healing in skin wounds.<sup>36</sup> These observations have led to the concept that LL-37 is a multifunctional effector molecule capable of directly killing pathogens, modulating the immune response, and promoting wound healing.<sup>14,37</sup>

The ocular surface stays remarkably free from infection despite constant exposure to a hostile microbial environment. This reality is attributable, in part, to a very robust innate immune response.<sup>38</sup> Recent studies have shown that the antimicrobial peptides human  $\alpha$ -defensin (hBD)-1, -2, and -3 are expressed by ocular surface epithelia and therefore are likely to contribute to the innate immune response.<sup>39-43</sup> The goals of the current study were (1) to determine whether LL-37, like  $\alpha$ -defensins, is secreted by corneal and conjunctival epithelia as part of the innate immune response, and (2) to assess *in vitro* the antimicrobial activity of LL-37 against major ocular bacterial and viral pathogens.

## MATERIALS AND METHODS

### Peptides

The antimicrobial peptide, LL-37, was purchased from American Peptide Company (Sunnyvale, CA, USA) and used in all the experiments. A scrambled peptide of LL-37<sup>35</sup> with the same amino acids ordered randomly was purchased from Global Peptide Services (Fort Collins, CO, USA).

## Human Corneal Epithelium

Human corneas were obtained from the Lions Eye Banks (Central Florida and Heartlands) and used in accordance with the tenets of the Declaration of Helsinki regarding the use of human tissue for research. The epithelium was scraped from each cornea (in the cases where a pair were available, then the tissue from both corneas was pooled) using a scalpel blade, then immediately placed in RNA lysis buffer (Qiagen, Valencia, CA, USA) for reverse transcription-polymerase chain reaction (RT-PCR) assays or snap frozen in liquid nitrogen and stored at -80°C for the immunoblot assays.

## Cell Culture

Primary cultures of human corneal epithelial cells (HCECs) were prepared from single or pairs of normal eye bank corneas based on the method described previously.<sup>42</sup> Following incubation in Dispase II (1.2 U/ml) for 4-5 hr at 37°C, the epithelial layer was scraped free from the underlying stroma with a no. 15 scalpel blade and transferred to a tube containing Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS) and centrifuged. The cell pellet was resuspended in EpiLife medium (Cascade Biologics, Portland, OR, USA), and a single cell suspension was obtained by titrating through a syringe fitted with a 22G needle. The cells were transferred to a culture flask coated with a mixture of fibronectin and collagen (FNC; AthenaES, Baltimore, MD, USA) containing 5 ml of serum-free EpiLife media with human corneal growth supplement (HCGS; Cascade Biologics). Primary cultured HCECs of passages 1 to 2 were used in the experiments.

Human conjunctival tissue from two donors was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA). Primary conjunctival epithelial cells were cultured as described previously.<sup>43</sup> Briefly, conjunctival tissue was incubated overnight at 4°C in a 1:1 (v/v) solution of EpiLife medium and dispase (20 U/ml). Using a scalpel blade, the epithelial cells were then scraped free and seeded into a FNC-coated flask with EpiLife media and HCGS. Primary-cultured conjunctival epithelial cells of passages 1 to 3 were used for the experiments.

## Reverse Transcription-Polymerase Chain Reaction

Total RNA from all cell samples was extracted using an RNeasy kit (Qiagen). Two hundred fifty nanograms of total RNA were used per RT-PCR reaction using a Superscript II (Invitrogen, Carlsbad, CA, USA) kit. Reactions containing normal human testis RNA (Clontech Laboratories, Palo Alto, CA, USA) or RNase free water in place of the RNA served as a positive control and negative control, respectively. Reverse transcription was performed at 50°C for 60 min. In some reactions, the reverse transcriptase was omitted (-RT control). After denaturation of the reverse transcriptase (94°C, 5 min), amplification of the cDNA was performed for 40 cycles: denaturation, 94°C for 50 s; annealing, 62°C for 30 s; extension 72°C for 1 min. The specific primers used were  $\alpha$ -actin (forward 5'-CCTCGCC-TTTGCCGATCC-3' and reverse 5'-GGATCTTCATGAGGTAGTCAGTC-3', 626 bp<sup>44</sup>) and LL-37/hCAP18 (forward 5'-ATCATTGCCAG-GTCCTCAG-3' and reverse 5'-GTCCCATACAC-CGCTTAC-3', 251 bp).<sup>45</sup> Products generated with these primers were sequenced (Seqwright, Houston, TX, USA) to confirm their identities. RT-PCR products were visualized on an agarose gel using an Alpha Imager gel documentation system (Alpha Innotec, San Leandro, CA, USA). HyperLadder I (Midwest Scientific, St. Louis, MO, USA) was used as the base pair marker.

## Immunoblot Analysis

Epithelial samples that had been snap frozen were used in immunoblots to detect LL-37 peptide. Each sample was homogenized in 100  $\mu$ l of ice-cold tris buffered saline (TBS, 150 mM NaCl,

20 mM Tris- HCl, pH 7.5). Cell lysate (25  $\mu$ g of total protein) was blotted directly onto a nitrocellulose membrane using a Bio-Dot Microfiltration apparatus (Life Science, Hercules, CA, USA). One nanogram of synthetic LL-37 peptide (American Peptide Company, Sunnyvale, CA, USA) was also blotted onto the membrane as a positive control. Nonspecific binding sites were blocked by incubation in 5% blotto, then the membrane was incubated with a rabbit anti- LL37 polyclonal antibody (donated by Dr. Lehrer, UCLA) diluted 1 in 5000 in 3% blotto. After an overnight incubation, the membranes were then incubated with a horseradish peroxidase linked second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1 in 10,000 in 3% blotto. Immunoreactivity was visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

### Antibacterial Assay

*Pseudomonas aeruginosa* (PA; ATCC 27853 and two clinical isolates, PA 2219 and PA 2212 from corneal scrapings of subjects with bacterial keratitis) were tested in this study. The ATCC strain tested is known to invade intact cornea and produce severe ocular infection in experimentally infected animal models of bacterial keratitis.<sup>46</sup> One single isolated PA colony was used to inoculate 5 ml of nutrient broth (NB) overnight at 37°C. Fifty microliters of this bacterial suspension were used to inoculate 50 ml of fresh NB, which was then incubated for 2.5 hr with vigorous shaking at 37°C to achieve mid-log phase growth. Twenty-five milliliters of the warm PA culture were centrifuged at 3100 g for 10 min, and the bacterial cell pellet was resuspended  $\times$  in cold phosphate buffer (PB, 8.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Optical density of the suspension was adjusted to 0.2 at 620 nm (approximately 10<sup>7</sup>cfu/ml) by adding an appropriate volume of PB. The antimicrobial assay procedure was adapted from that described by Tomita *et al.*<sup>47</sup> Reaction mixtures (final volume 50 ml) containing 10 ml of 10<sup>7</sup>cfu/ml PA and 5 ml LL-37 diluted in PB (final concentration 0.05, 0.1, 0.5, 1, 10, 25, 50, and 100  $\mu$ g/ $\mu$ l) were incubated at 37°C for 2 hr with vigorous shaking. In each experiment, reaction mixtures containing 5  $\mu$ l of 0.01% acetic acid, the vehicle for diluting LL-37, acted as a control. At the end of the incubation, serial dilutions of each reaction mixture were used to inoculate NB agar plates. Samples (10  $\mu$ l) were spread evenly over the surface of the plates using sterile glass spreaders. After incubation at 37°C for 24 hr, the plates were placed on a lightboard and a digital image captured using an Alpha Imager documentation system. The number of colonies was counted using the colony count software of the Alpha Imager. The percentage of bacteria killed by LL- 37 was calculated using the following equation: Killing (%) = ([number of PA colonies]<sub>control</sub>[number of PA colonies]-  $\times$  LL-37)/[number of PA colonies]<sub>control</sub> 100%. EC<sub>50</sub> values were calculated using GraphPad Prism4 software (GraphPad Software, San Diego, CA, USA).

Additional experiments were also performed to test the antimicrobial activity of LL-37 against *Staphylococcus aureus* (SA; ATCC 29213) and *Staphylococcus epidermidis* (SE; ATCC 155). Testing conditions in these experiments were identical to those described for PA with the exception of replacing NB with trypticase soy broth.

### MTT Cytotoxicity Assay

A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Ham's F12 with 10% FBS and passed by standard methods using trypsin/EDTA. For cytotoxicity assays, the cells (p89, 96) were grown to confluency in 96-well plates. LL-37 and scrambled LL- 37<sup>35</sup> were diluted in culture medium with or without 10% FBS to give final concentrations of 50, 5, and 0.5  $\mu$ g/ml. These concentrations matched those of the peptides during the adsorption phase of the antiviral assay. The cells were then exposed to the diluted peptides for 3 hr. Controls received either culture media alone or culture medium with 10% FBS. Positive controls were incubated with 0.002% benzalkonium chloride for 15 min. Four to five replicates were performed for each condition. Cytotoxicity was assessed

using an MTT assay kit (Chemicon International, Temecula, CA, USA) as per the manufacturer's instructions.

### Antiviral Assay: Direct Inactivation Time-Kill Assay

A time-kill direct inactivation assay was used to determine the antiviral inhibitory activity of the antimicrobial peptides LL-37 and a scrambled peptide<sup>35</sup> on two different DNA viruses: herpes simplex virus type 1 (McKrae strain) and several adenovirus serotypes (Ad3, Ad5, Ad8, Ad19) isolated from epidemic keratoconjunctivitis (EKC) patients presenting to The Charles T. Campbell Ophthalmic Microbiology Laboratory (Pittsburgh, PA, USA). In a single tube, each virus sample ( $\sim 10^4$  pfu) was incubated directly with LL-37 (500  $\mu\text{g/ml}$ , 111  $\mu\text{M}$ ), scrambled peptide (500  $\mu\text{g/ml}$ , 111  $\mu\text{M}$ ), or PBS in a 37°C water bath. At 1 and 4 hr, 35  $\mu\text{l}$  aliquots were removed from the virus-peptides and virus-PBS incubation mixtures and were diluted 1:10 in fresh ice-cold (0°C) media containing 10% fetal bovine serum to dilute the remaining virus and inactivate the peptides.<sup>48,49</sup> The mixtures were further serially diluted and titrated the same day on A549 cells as previously described.<sup>50</sup> In order to evaluate reproducibility, a series of 6 independent experiments comprising each virus assay were carried out at different times and the data analyzed statistically using one-way analysis of variance (ANOVA) (Minitab 12 for Windows, Minitab, State College, PA, USA).

## RESULTS

### LL-37 Expression in Human Ocular Surface Epithelia

RT-PCR was performed to study LL-37/hCAP18 mRNA expression in human corneal and conjunctival epithelial cells (Fig. 1A). LL-37/hCAP18 mRNA was constitutively expressed in six of seven scraped corneal epithelial samples and in all primary cultures of corneal (n = 8) and conjunctival (n 2) epithelial cells tested. Immunoblotting was performed to study LL-37 peptide expression by human corneal epithelium. The results from four representative samples are shown in Figure 1B. LL-37 peptide was present in both scraped epithelial samples (n 6) and primary cultured epithelial cells (n=3)

### Antibacterial Activity LL-37 Against *Pseudomonas Aeruginosa*, *Staphylococcus Aureus*, and *Staphylococcus Epidermidis*

Antibacterial assays were performed to study the activity of LL-37 against PA (ATCC 27853 and two clinical isolates). As shown in Figure 2, LL-37 inhibited growth of all three PA strains in a concentration-dependent manner. The  $\text{EC}_{50}$  was 2.8 1.3 (n 3),  $\pm 1.9 \pm 1.3$  (n = 3), and  $3.6 \pm$  (n = 3)  $\mu\text{g/ml}$  for ATCC 2.1 27853 and the two clinical strains (PA 2219 and PA 2212), respectively. At 100  $\mu\text{g/ml}$ , LL-37 was completely bactericidal for all strains of PA. LL-37 also showed potent bactericidal activity against SA and SE with  $\text{EC}_{50}$  values being  $1.6 \pm 1.5$  (n = 3) and 1.31.9 (n 3)  $\mu\text{g/ml}$ , respectively.

### LL-37 and Scrambled LL-37 do not Show Toxicity to A549 Cells

A cell survival assay was performed to ensure that LL- 37 and scrambled LL-37 were not toxic to A549 cells at the concentrations tested in the antiviral assay (Fig. 3). Exposure to benzalkonium chloride, the positive control, caused significant A549 cell death ( $p \geq 0.001$ , Student's *t* test), whereas neither LL-37 nor the scrambled peptide showed any toxicity toward A549 cells at any of the concentrations tested in the presence of 10% FBS (n = 2). Identical results were obtained when the experiment was carried out in the absence of 10% FBS (data not shown).



### Antiviral Activity of LL-37 and Scrambled LL-37 Against HSV-1 and Clinical Ad Serotypes

As LL-37 and the scrambled peptide were shown to be nontoxic in A549 cells at the concentration tested, the antiviral assays results shown in Figures 4 and 5 could be properly ascribed to the peptides' intrinsic inhibitory capabilities. For HSV-1 (Fig. 4), LL-37 produced a highly significant ( $p < 0.001$  ANOVA)  $\geq 2$  log reduction in HSV-1 titers compared to PBS and scrambled peptide at 4 hr. Although the scrambled peptide demonstrated limited inhibition of HSV-1 relative to the PBS control ( $p < 0.001$ ), it was significantly less ( $p < 0.001$ ) than that of LL-37.

Among the Ad serotypes tested (Ad8, Ad5, Ad3) at 4 hr, all demonstrated a trend toward showing an inhibitory effect by LL-37 (Fig. 4). However, only Ad19 demonstrated statistically significant ( $p < 0.001$  ANOVA) reduction in titers compared to the scrambled peptide and PBS controls. Like for HSV-1, the scrambled peptide again demonstrated limited inhibition of Ad19 relative to the PBS control ( $p < 0.001$ ), but was significantly less ( $p < 0.001$ ) than that of the LL-37.

The time-kill curves comparing HSV-1 and Ad19 over 4 hr are summarized in Figure 5. For HSV-1, LL-37 produced a rapid, highly significant ( $p < 0.007$ ) 2 log reduction in viral titers within 1 hr, while for Ad19, there was a much slower progressive reduction in titers to reach a maximum effect at 4 hr. These differences in the slopes of the kill curves suggest a possible different mechanism of inhibitory action of LL-37 for an enveloped virus (HSV-1) compared to a nonenveloped virus (Ad19).<sup>48</sup>

## DISCUSSION

LL-37 is a multifunctional peptide with many important biological activities: antimicrobial activity, chemoattraction, dendritic cell differentiation, mast cell degranulation, cytokine secretion, angiogenesis stimulation, and wound healing.<sup>2,3,14,18,31-34,36,37</sup> The current study reports for the first time that the epithelial cells of both the cornea and conjunctiva express this important effector of innate immunity. This discovery was initially reported in 2003<sup>51</sup> and has recently been confirmed in a report by Dua *et al.*<sup>52</sup> Our discovery of LL-37 as a part of ocular mucosal defense complements previous studies that have demonstrated that  $\alpha$ -defensins hBD-1, -2, and -3 are also secreted by conjunctival and corneal epithelium.<sup>39-43,53</sup> Taken together, these findings suggest that the ocular surface must be added to the growing list of mucosal surfaces throughout the body where multifunctional cationic peptides operate as part of innate immunity.

LL-37 may have several activities at the ocular surface. It is likely to be involved in direct killing of invading pathogens and through its various effector activities may be involved in regulating the ocular surface immune response and wound healing. Indeed, preliminary studies from our laboratory do show that LL-37 can stimulate corneal epithelial cell migration.<sup>54</sup> In this report, we focused our attention on the role of LL-37 as an ocular antimicrobial peptide and studied its effectiveness against common ocular pathogens.

We investigated the antimicrobial activity of synthetic LL-37 against the Gram-positive (SE, SA) and Gram-negative (PA) organisms most often responsible for bacterial keratitis and the pathogens most frequently associated with keratitis in contact lens wear.<sup>55-57</sup> We found LL-37 to have potent antibacterial activity against both a laboratory strain and clinical isolates of PA. Our EC<sub>50</sub> values of approximately 2-4  $\mu\text{g/ml}$  compare well with published studies of the effectiveness of LL-37 against other PA strains.<sup>20,27,28,58,59</sup> LL-37 also demonstrated potent antibacterial activity against the most common Gram-positive keratitis-producing organisms: SE (mean EC<sub>50</sub> 1.3  $\mu\text{g/ml}$ ) and SA (mean EC<sub>50</sub> 1.6  $\mu\text{g/ml}$ ).

To explore a possible broader antimicrobial role of LL-37 in defense of the ocular surface, we also studied the effectiveness of the peptide against ocular viral pathogens in a direct inactivation assay. We report for the first time that LL-37 demonstrates significant antiviral inhibitory activity (>98% inhibition) against HSV-1, the leading viral cause of corneal blindness in industrialized countries.<sup>60</sup> In a previous study, Yasin *et al.* reported limited anti-HSV-1 activity for LL-37 compared to two other more potent antimicrobial peptides, brevidin and indolicidin, in a MTT assay.<sup>29</sup> The differences in results between the studies may be explained by differences in HSV-1 virus strains, host cells, incubation times, peptide concentrations, and the protocol for the antiviral assays used.

Additionally, we report for the first time that LL-37 demonstrated statistically significant inhibitory activity *in vitro* against Ad19, a major cause of conjunctivitis and epidemic keratoconjunctivitis in local and global epidemics.<sup>61</sup> Previous studies have reported that certain defensins have antiviral activity against genetically engineered adenovirus type 5 vectors used in gene therapy,<sup>62,63</sup> but there are no earlier reports that any antimicrobial peptides (defensins or LL-37) can inhibit wild-type pathogenic adenoviruses. Our observation represents a “proof-of-principle” that an antimicrobial peptide can inhibit at least one nonenveloped virus under experimental conditions by direct inactivation. Serotype differences in the susceptibility to LL-37 may be related to the known structural differences among different Ad serotypes in the hypervariable region of the hexon capsid<sup>64</sup> and the penton fiber.<sup>65</sup>

We evaluated specificity in our *in vitro* antiviral assay by comparing the inhibitory activity of the LL-37 to a scrambled peptide (same size, same amino acids in a different order) that had previously demonstrated no activity in promoting angiogenesis<sup>35</sup> or corneal epithelial cell migration.<sup>51</sup> We conclude that “reasonable” specificity was demonstrated in our studies as limited inhibition was achieved by the scrambled peptide against HSV-1 and Ad19. Notably for both viruses, the effect of the scrambled peptide was significantly less than for LL-37 (Fig. 4). We explain the limited activity of the scrambled peptide on the fact that the antimicrobial function of peptides is known to be multivariate being dependent not only on size and sequence but also charge, degree of structuring (helicity), overall hydrophobicity, amphipathicity, and the angles subtended by hydrophobic and hydrophilic surfaces of a helical molecule.<sup>66</sup> Therefore, size and amino acid sequence alone appeared to be insufficient to demonstrate complete specificity for the two different viruses tested.

The current study suggests possible differences in the mechanism of inhibition based on the slopes of the LL-37 curves in the time-kill assays. The rapid killing of HSV-1 (<1 hr) suggests that permeabilization of the external lipid membrane is the most likely mechanism. This mechanism has been previously proposed for all cationic peptides (e.g., defensins) against HSV-1 and other enveloped viruses, bacteria, and fungi.<sup>2,37,48,58,67,68</sup> Furthermore, the antiviral activity of LL-37 against vaccinia virus<sup>30</sup> has also been attributed to its direct disruptive action on the viral envelope. Interestingly, recent studies of defensins<sup>69,70</sup> and other peptides<sup>71,72</sup> against HSV and defensins against HIV<sup>73-75</sup> indicate that prevention of viral entry is another important mechanism of peptide inhibitory action against these enveloped viruses.

Unlike enveloped viruses, all adenoviridae lack a surrounding host cell-derived lipid membrane suggesting that the direct killing through permeabilization of the viral envelope cannot be the operative mechanism. Alternative mechanisms such as disruption of the adenovirus particle (detergent-effect) and/or blockage of viral entry (attachment/endocytosis) into the cell remain possibilities requiring further clarification. Further studies are planned with LL-37 at different times in the adenovirus life cycle to further delineate extracellular and possible intracellular mechanisms of inhibition.

The evolutionary selection of diverse antimicrobial peptides based on a recently described universal multidimensional signature (i.e., a common three-dimensional structure) appears to be an essential component of innate immunity.<sup>76</sup> During the past 2.6 billion years, most life forms (bacteria, fungi, plants, invertebrates, and vertebrates) have successfully used these peptides to defend against a hostile surrounding microbial world. The rapid doubling time of bacteria has been considered by many to be the driving force for the evolution of innate immunity, as the threat is constant and the speed of host response must be appropriate to the pathogen.<sup>68</sup> For this reason, any antiviral activity demonstrated by antimicrobial peptides against enveloped viruses has been deemed secondary to the common mechanism of action, permeabilization of membranes. The recent observations that defensins can actually prevent entry of HSV<sup>69,70</sup> and HIV,<sup>73-75</sup> both enveloped viruses, and our observation here that LL-37 is effective against the nonenveloped virus Ad19 strongly support the notion that the antiviral activity of antimicrobial peptides is not coincidental but an important part of innate immunity that concurrently evolved with its antibacterial and antifungal capabilities. Although the time frame of virus replication is admittedly longer (20+ hr) than bacteria, the optimal strategy to limit these obligate intracellular parasites is to destroy them rapidly externally and/or prevent their entry into uninfected host cells. Like bacteria, this strategy also requires a rapid host response. Therefore, we surmise that evolution would likely select for rapidly mobilized effector molecules with antiviral capabilities (e.g., antimicrobial peptides) to protect host cells from invasion, colonization (HSV-1 latency), and death. A number of *in vivo* studies have shown that antimicrobial peptides (defensins and cathelicidins) are effective in defense against bacterial infections.<sup>69,70,77-79</sup> Whether biologically significant antiviral activity of these peptides can be expanded beyond enveloped viruses to include nonenveloped viruses remains a distinct possibility, but further confirmation is required.

#### ACKNOWLEDGMENTS

This work was supported by EY 008227 (Y.J.G.), Core Grant for Vision Research EY08098, The Eye and Ear Foundation of Pittsburgh, Research for the Prevention of Blindness, University of Houston Gear grant and EY-13175 (A.M.M.). The authors are grateful to Lions Eye Banks (Central Florida and Heartlands) for supplying the human corneas and to Dr. Robert Lehrer (UCLA) for his generous donation of LL-37 antibodies. Thanks also to Dr. Bradley Mitchell (Baylor College of Medicine) for providing the clinical PA isolates, Srihari Narayanan for culturing conjunctival epithelial cells, and Daniele Jean for assisting with the antibacterial assay.

#### REFERENCES

- [1]. Kaufmann, H.; Medzhitov, R.; Gordon, S., editors. *The Innate Immune Response To Infection*. ASM Press; Washington, DC: 2004. p. 465
- [2]. Bals R, Wilson JM. Cathelicidins—a family of multifunctional antimicrobial peptides. *Cell Mol Life Sci* 2003;60:711–720. [PubMed: 12785718]
- [3]. Gallo RL, Nizet V. Endogenous production of antimicrobial peptides in innate immunity and human disease. *Curr Allergy Asthma Rep* 2003;3:402–409. [PubMed: 12906776]
- [4]. Dempsey PW, Vaidya SA, Cheng G. The art of war: innate and adaptive immune responses. *Cell Mol Life Sci* 2003;60:2604–2621. [PubMed: 14685686]
- [5]. Beutler B. Innate immunity: an overview. *Mol Immunol* 2004;40:845–859. [PubMed: 14698223]
- [6]. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol* 1998;16:82–88. [PubMed: 9487736]
- [7]. Schroder JM. Epithelial peptide antibiotics. *Biochem Pharmacol* 1999;57:121–134. [PubMed: 9890560]
- [8]. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389–395. [PubMed: 11807545]
- [9]. Brogden KA, Ackermann M, McCray PB Jr, Tack BF. Antimicrobial peptides in animals and their role in host defences. *Int J Antimicrob Agents* 2003;22:465–478. [PubMed: 14602364]
- [10]. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003;3:710–720. [PubMed: 12949495]

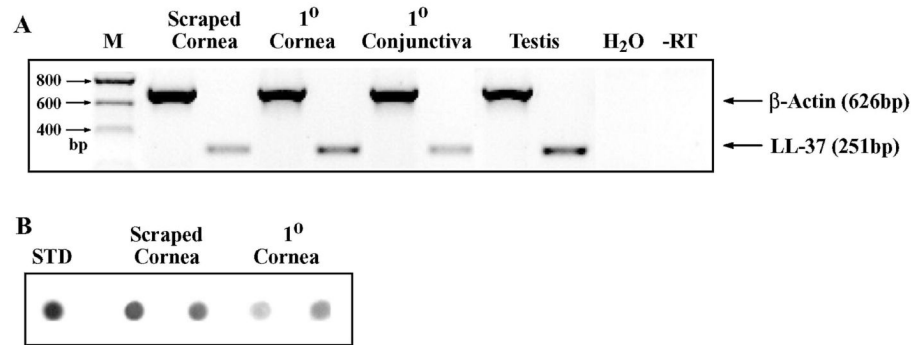


- [11]. van 't Hof W, Veerman EC, Helmerhorst EJ, Amerongen AV. Antimicrobial peptides: properties and applicability. *Biol Chem* 2001;382:597–619. [PubMed: 11405223]
- [12]. Shai Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers* 2002;66:236–248. [PubMed: 12491537]
- [13]. Scott MG, Hancock RE. Cationic antimicrobial peptides and their multifunctional role in the immune system. *Crit Rev Immunol* 2000;20:407–431. [PubMed: 11145218]
- [14]. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* 2004;22:181–215. [PubMed: 15032578]
- [15]. Yang D, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* 2002;23:291–296. [PubMed: 12072367]
- [16]. Gennaro R, Zanetti M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers* 2000;55:31–49. [PubMed: 10931440]
- [17]. Nagaoka I, Hirota S, Yomogida S, Ohwada A, Hirata M. Synergistic actions of antibacterial neutrophil defensins and cathelicidins. *Inflamm Res* 2000;49:73–79. [PubMed: 10738945]
- [18]. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004;75:39–48. [PubMed: 12960280]
- [19]. De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPR1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000;192:1069–1074. [PubMed: 11015447]
- [20]. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci USA* 1998;95:9541–9546.
- [21]. Frohm M, Agerberth B, Ahangari G, et al. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 1997;272:15258–15263. [PubMed: 9182550]
- [22]. Frohm Nilsson M, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M. The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect Immun* 1999;67:2561–2566. [PubMed: 10225921]
- [23]. Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 1997;90:2796–2803. [PubMed: 9326247]
- [24]. Dorschner RA, Pestonjamas VK, Tamakuwala S, et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A *Streptococcus*. *J Invest Dermatol* 2001;117:91–97. [PubMed: 11442754]
- [25]. Sorensen OE, Follin P, Johnsen AH, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 2001;97:3951–3959. [PubMed: 11389039]
- [26]. Zaiou M, Nizet V, Gallo RL. Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *J Invest Dermatol* 2003;120:810–816. [PubMed: 12713586]
- [27]. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob Agents Chemother* 1998;42:2206–2214. [PubMed: 9736536]
- [28]. Travis SM, Anderson NN, Forsyth WR, et al. Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect Immun* 2000;68:2748–2755. [PubMed: 10768969]
- [29]. Yasin B, Pang M, Turner JS, et al. Evaluation of the inactivation of infectious herpes simplex virus by host-defense peptides. *Eur J Clin Microbiol Infect Dis* 2000;19:187–194. [PubMed: 10795591]
- [30]. Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol* 2004;172:1763–1767. [PubMed: 14734759]

- [31]. Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol* 2001;31:1066–1075. [PubMed: 11298331]
- [32]. Niyonsaba F, Iwabuchi K, Someya A, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* 2002;106:20–26. [PubMed: 11972628]
- [33]. Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol* 2002;169:3883–3891. [PubMed: 12244186]
- [34]. Davidson DJ, Currie AJ, Reid GS, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J Immunol* 2004;172:1146–1156. [PubMed: 14707090]
- [35]. Koczulla R, von Degenfeld G, Kupatt C, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 2003;111:1665–1672. [PubMed: 12782669]
- [36]. Heilborn JD, Nilsson MF, Kratz G, et al. The cathelicidin antimicrobial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol* 2003;120:379–389. [PubMed: 12603850]
- [37]. Koczulla AR, Bals R. Antimicrobial peptides; current status and therapeutic potential. *Drugs* 2003;63:389–406. [PubMed: 12558461]
- [38]. Sack RA, Nunes I, Beaton A, Morris C. Host-defense mechanism of the ocular surfaces. *Biosci Rep* 2001;21:463–480. [PubMed: 11900322]
- [39]. Haynes RJ, Tighe PJ, Dua HS. Innate defence of the eye by antimicrobial defensin peptides. *Lancet* 1998;352:451–452. [PubMed: 9708756]
- [40]. Hattenbach LO, Gumbel H, Kippenberger S. Identification of beta-defensins in human conjunctiva. *Antimicrob Agents Chemother* 1998;42:3332. [PubMed: 10049242]
- [41]. McNamara NA, Van R, Tuchin OS, Fleiszig SM. Ocular surface epithelia express mRNA for human beta defensin-2. *Exp Eye Res* 1999;69:483–490. [PubMed: 10548468]
- [42]. McDermott AM, Redfern RL, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signaling pathways mediate IL-1beta stimulation of hbD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2003;44:1859–1865. [PubMed: 12714616]
- [43]. Narayanan S, Miller WL, McDermott AM. Expression of human beta-defensins in conjunctival epithelium: relevance to dry eye disease. *Invest Ophthalmol Vis Sci* 2003;44:3795–3801. [PubMed: 12939294]
- [44]. Raff T, van der Giet M, Endemann D, Wiederholt T, Paul M. Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques* 1997;23:456–460. [PubMed: 9298216]
- [45]. Paulsen FP, Pufe T, Schaudig U, et al. Detection of natural peptide antibiotics in human nasolacrimal ducts. *Invest Ophthalmol Vis Sci* 2001;42:2157–2163. [PubMed: 11527925]
- [46]. O'Callaghan RJ, Engel LS, Hobden JA, Callegan MC, Green LC, Hill JM. *Pseudomonas* keratitis. The role of an uncharacterized exoprotein, protease IV, in corneal virulence. *Invest Ophthalmol Vis Sci*. 1996;37:534–543.
- [47]. Tomita T, Hitomi S, Nagase T, et al. Effect of ions on antibacterial activity of human beta defensin 2. *Microbiol Immunol* 2000;44:749–754. [PubMed: 11092238]
- [48]. Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granulocyte defensins. *J Virol* 1986;60:1068–1074. [PubMed: 3023659]
- [49]. Bartlett KH, McCray PB Jr, Thorne PS. Reduction in the bactericidal activity of selected cathelicidin peptides by bovine calf serum or exogenous endotoxin. *Int J Antimicrob Agents* 2004;23:606–612. [PubMed: 15194132]
- [50]. Gordon YJ, Romanowski EG, Araullo-Cruz T. HPMPC, a broadspectrum topical antiviral agent, inhibits herpes simplex virus type 1 replication and promotes healing of dendritic keratitis in the New Zealand rabbit ocular model. *Cornea* 1994;13:516–520. [PubMed: 7842711]
- [51]. Huang L, Proske RJ, McDermott AM. Expression of the peptide antibiotic LL-37/hCAP18 (Cathelicidin) by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2003;44:1335. ARVO e-abstract

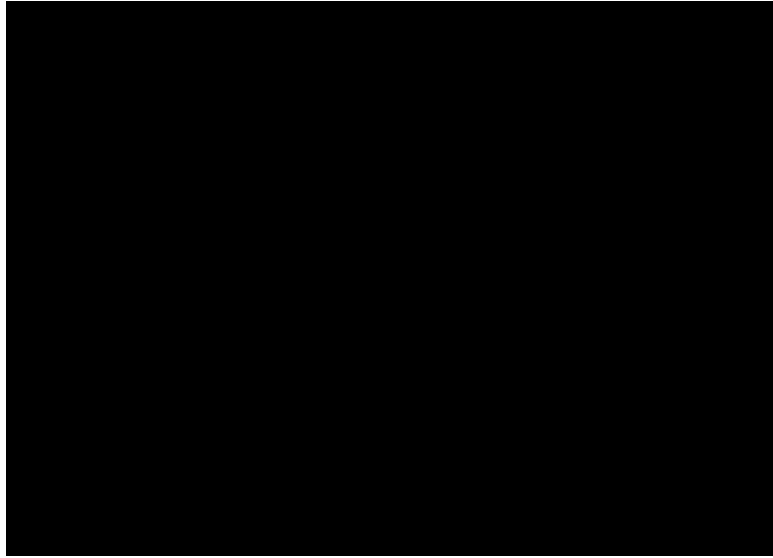
- [52]. McIntosh RS, Cade JE, Al-Abed M, Shanmuganathan V, Gupta R, Bhan A, Tighe PJ, Dua HS. The spectrum of antimicrobial peptide expression at the ocular surface. *Invest Ophthalmol Vis Sci* 2005;46:1379–1385. [PubMed: 15790905]
- [53]. Lehmann OJ, Hussain IR, Watt PJ. Investigation of beta defensin gene expression in the ocular anterior segment by semiquantitative RT-PCR. *Br J Ophthalmol* 2000;84:523–526. [PubMed: 10781518]
- [54]. Huang L, Proske RJ, McDermott AM. Functional roles of the epithelial-derived antimicrobial-derived peptide LL-37 at the ocular surface. *Invest Ophthalmol Vis Sci* 2004;45:4933. ARVO e-abstract
- [55]. Schaefer F, Bruttin O, Zografos L, Guex-Crosier Y. Bacterial keratitis: a prospective clinical and microbiological study. *Br J Ophthalmol* 2001;85:842–847. [PubMed: 11423460]
- [56]. Bourcier T, Thomas F, Borderie V, Chaumeil C, Laroche L. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol* 2003;87:834–838. [PubMed: 12812878]
- [57]. Mela EK, Giannelou IP, Koliopoulos JX, Gartaganis SP, John KX, Sotirios GP. Ulcerative keratitis in contact lens wearers. *Eye Contact Lens* 2003;29:207–209. [PubMed: 14555893]
- [58]. Bucki R, Pastore JJ, Randhawa P, Vegners R, Weiner DJ, Janney PA. Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob Agents Chemother* 2004;48:1526–1533. [PubMed: 15105101]
- [59]. Saiman L, Tabibi S, Starner TD, et al. Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. *Antimicrob Agents Chemother* 2001;45:2838–2844. [PubMed: 11557478]
- [60]. Pepose, JS.; Leib, DL.; Stuart, PM.; Easty, DL. *Ocular Immunity and Infection*. Pepose, JS.; Holland, GN.; Wilhelmus, KR., editors. St. Louis; Mosby: 1996. p. 905-932.
- [61]. Gordon, YJ.; Aoki, K.; Kinchington, PR. *Ocular Immunity and Infection*. Pepose, JS.; Holland, GN.; Wilhelmus, KR., editors. St. Louis; Mosby: 1996. p. 877-894.
- [62]. Gropp R, Frye M, Wagner TO, Bargon J. Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. *Hum Gene Ther* 1999;10:957–964. [PubMed: 10223729]
- [63]. Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther* 1997;8:37–44. [PubMed: 8989993]
- [64]. Shimada Y, Ariga T, Tagawa Y, Aoki K, Ohno S, Ishiko H. Molecular diagnosis of human adenoviruses d and e by a phylogeny-based classification method using a partial hexon sequence. *J Clin Microbiol* 2004;42:1577–1584. [PubMed: 15071007]
- [65]. Liebermann H, Lotz K, Seidel W. Mapping of epitopes on the fiber knobs of human adenovirus serotypes 8 and 15. *Intervirology* 2002;45:59–66. [PubMed: 11937773]
- [66]. Giangaspero A, Sandri L, Tossi A. Amphipathic alpha helical antimicrobial peptides. *Eur J Biochem* 2001;268:5589–5600. [PubMed: 11683882]
- [67]. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* 2003;55:27–55. [PubMed: 12615953]
- [68]. Boman HG. Antibacterial peptides: basic facts and emerging concepts. *J Intern Med* 2003;254:197–215. [PubMed: 12930229]
- [69]. Sinha S, Cheshenko N, Lehrer RI, Herold BC. NP-1, a rabbit alpha-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. *Antimicrob Agents Chemother* 2003;47:494–500. [PubMed: 12543649]
- [70]. Yasin B, Wang W, Pang M, et al. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J Virol* 2004;78:5147–5156. [PubMed: 15113897]
- [71]. Bultmann H, Brandt CR. Peptides containing membrane-transiting motifs inhibit virus entry. *J Biol Chem* 2002;277:36018–36023. [PubMed: 12130644]
- [72]. Bultmann H, Busse JS, Brandt CR. Modified FGF4 signal peptide inhibits entry of herpes simplex virus type 1. *J Virol* 2001;75:2634–2645. [PubMed: 11222686]

- [73]. Quinones-Mateu ME, Lederman MM, Feng Z, et al. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *AIDS* 2003;17:F39–48. [PubMed: 14571200]
- [74]. Wang W, Owen SM, Rudolph DL, et al. Activity of alpha-and theta-defensins against primary isolates of HIV-1. *J Immunol* 2004;173:515–520. [PubMed: 15210812]
- [75]. Zhang L, Yu W, He T, et al. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 2002;298:995–1000. [PubMed: 12351674]
- [76]. Yount NY, Yeaman MR. Multidimensional signatures in antimicrobial peptides. *Proc Natl Acad Sci USA* 2004;101:7363–7368.
- [77]. Wilson CL, Ouellette AJ, Satchell DP, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 1999;286:113–117. [PubMed: 10506557]
- [78]. Nizet V, Ohtake T, Lauth X, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;414:454–457. [PubMed: 11719807]
- [79]. Moser C, Weiner DJ, Lysenko E, Bals R, Weiser JN, Wilson JM. beta-Defensin 1 contributes to pulmonary innate immunity in mice. *Infect Immun* 2002;70:3068–3072. [PubMed: 12010999]

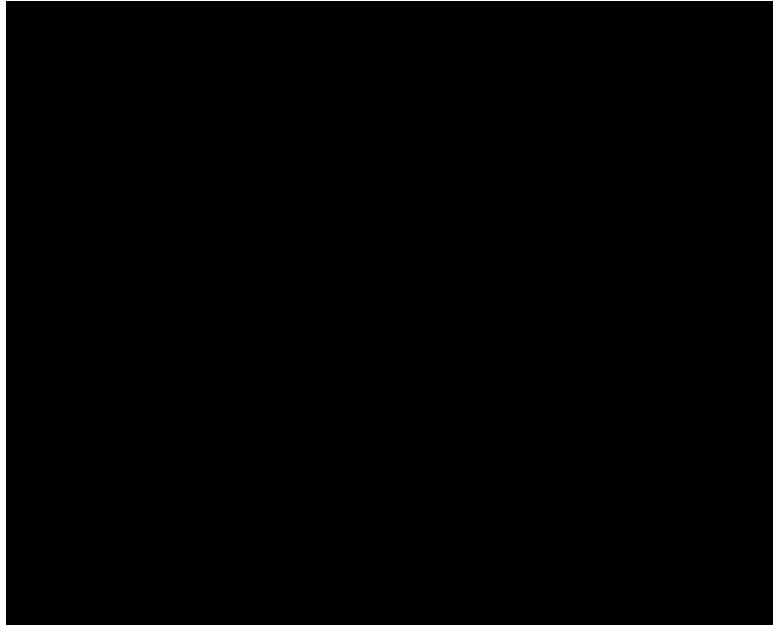
**FIGURE 1.**

Expression of LL-37 by ocular surface epithelia. (A) Expression of LL-37/hCAP18 mRNA by RT-PCR. The figure shows representative results for M, base pair size marker; scraped cornea, scraped human corneal epithelium (n = 6); 1° cornea, primary cultured human corneal epithelial cells (n=8); 1° conjunctiva, primary cultured human conjunctival epithelial cells (n=2); testis=positive control; H<sub>2</sub>O and -RT, water and -RT negative controls, respectively. (B) Expression of LL-37 protein by immunostaining. The figure shows representative results for, STD (standard), 1 ng LL-37 synthetic peptide; scraped cornea, 25 μg cellular protein from scraped human corneal epithelium (n = 6); 1° cornea, 25 μg cellular protein from primary cultured human corneal epithelial cells (n = 3). All samples were handled independently, and n is the number of different donors from which cells were obtained or cultures prepared.

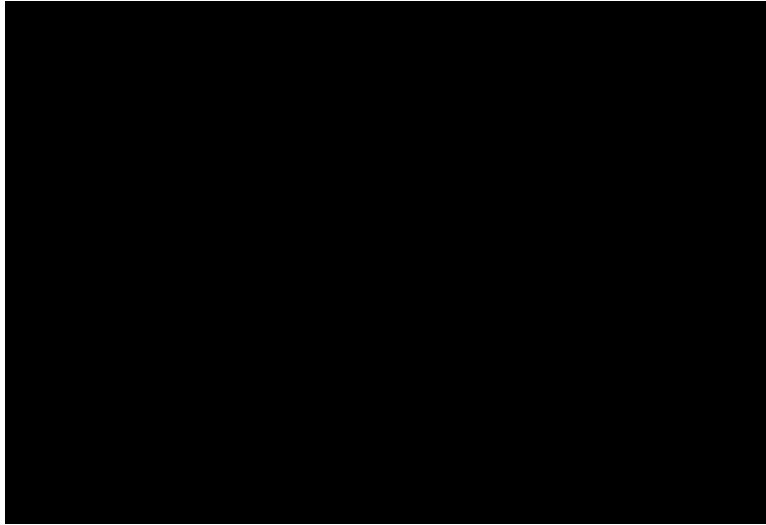


**FIGURE 2.**

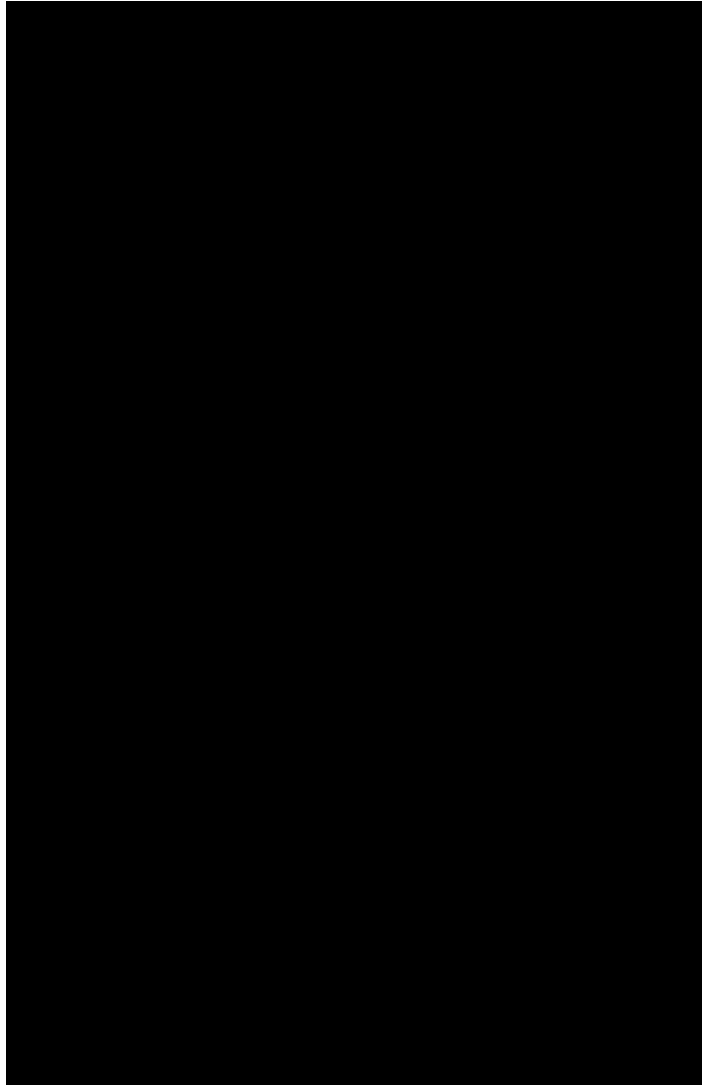
Antibacterial effect of LL-37 against *Pseudomonas aeruginosa*:  $10^7$  cfu/ml PA (ATCC 27853 and two clinical isolates) were incubated with LL-37 (0.05, 0.1, 0.5, 1, 10, 25, 50, and 100 $\mu$ g/ml) for 2 hr. The graph shows the average data of three experiments against each PA strain. The EC<sub>50</sub> values were 2.8  $\pm$  1.3 (ATCC 27853), 1.9 $\pm$ 1.3, and 3.6 $\pm$ 2.1  $\mu$ g/ml for each of the  $\pm$ two clinical PA strains (PA 2219 and PA 2212, respectively).

**FIGURE 3.**

LL-37 and scrambled LL-37 are not toxic to A549 cells. An MTT-based assay was used to assess cytotoxicity under conditions mimicking those of the antiviral assay. BAC, benzalkonium chloride; C, control; sLL-37, scrambled LL-37. Data shown are from a representative experiment repeated twice and are the mean  $\pm$ SD of 4-5 replicates per condition. \*  $p \leq 0.001$  compared to control. Identical results were obtained if the experiment was carried out in the absence of 10% FBS (data not shown).

**FIGURE 4.**

Antiviral activity of LL-37 against HSV-1 and clinical Ad serotypes. HSV-1: \*LL-37 produced a highly significant  $\geq 2$  log reduction in HSV-1 titers compared to PBS and scrambled peptide at 4 hr ( $p < 0.001$  ANOVA). \*\*Scrambled peptide inhibited HSV-1 more than PBS control ( $p < 0.001$ ), but less than LL-37 ( $p < 0.001$ ). ( $n = 6$  experiments). Ad19 \*LL-37 significantly reduced Ad19 titers compared to the scrambled peptide and PBS controls ( $p < 0.001$ , ANOVA). \*\*Scrambled peptide inhibited Ad19 titers more than the PBS control ( $p < 0.001$ ), but less than LL-37 ( $p < 0.001$ ). Note: Ad8, Ad5, and Ad3 all demonstrated a trend toward showing an antiviral inhibitory effect by LL-37 ( $n = 6$  experiments).



**FIGURE 5.** Kill curves of LL-37 against HSV-1 and Ad19. At 1 hr, LL-37 produced a rapid, highly significant ( $p < 0.007$ ) 2 log reduction in HSV-1 titers (A) compared to the much slower continuous reduction in titers for Ad19 (B) ( $p < 0.001$ ). These differences in the slopes of the kill curves over 4 hr suggest a possible different mechanism of inhibitory action of LL-37 for an enveloped virus (HSV-1) compared to a nonenveloped virus (Ad19). Asterisks indicate statistically significant differences for LL-37 (\*) and scrambled peptide (\*\*), respectively, compared to PBS controls at different time points. See “Results” for details ( $n = 6$  experiments).