Cathelicidin deficiency predisposes to eczema herpeticum

Michael D. Howell, PhD,[a] Andreas Wollenberg, MD,[b] Richard L. Gallo, MD, PhD,[c] Michael Flaig, MD,[b] Joanne E. Streib, BA,[a] Cathy Wong, BS,[c] Tatjana Pavicic, MD,[b] Mark Boguniewicz, MD,[a] and Donald Y. M. Leung, MD, PhD[a] *Denver, Colo, Munich, Germany, and San Diego, Calif*

Background: The cathelicidin family of antimicrobial peptides is an integral component of the innate immune response that exhibits activity against bacterial, fungal, and viral pathogens. Eczema herpeticum (ADEH) develops in a subset of patients with atopic dermatitis (AD) because of disseminated infection with herpes simplex virus (HSV).
Objective: This study investigated the potential role of cathelicidins in host susceptibility to HSV infection.
Methods: Glycoprotein D was measured by means of real-time RT-PCR as a marker of HSV replication in skin biopsy specimens and human keratinocyte cultures. Cathelicidin expression was evaluated in skin biopsy specimens from patients with AD (n = 10) without a history of HSV skin infection and from patients with ADEH (n = 10).
Results: The cathelicidin peptide LL-37 (human cathelicidin) exhibited activity against HSV in an antiviral assay, with significant killing ($P < .001$) within the physiologic range. The importance of cathelicidins in antiviral skin host defense was confirmed by the observation of higher levels of HSV-2 replication in cathelicidin-deficient ($Cnlp^{-/-}$) mouse skin (2.6 ± 0.5 pg HSV/pg GAPDH, $P < .05$) compared with that seen in skin from their wild-type counterparts (0.9 ± 0.3). Skin from patients with ADEH exhibited significantly ($P < .05$) lower levels of cathelicidin protein expression than skin from patients with AD. We also found a significant inverse correlation between cathelicidin expression and serum IgE levels ($r^2 = 0.46$, $P < .05$) in patients with AD and patients with ADEH.
Conclusion: This study demonstrates that the cathelicidin peptide LL-37 possesses antiviral activity against HSV and demonstrates the importance of variable skin expression of cathelicidins in controlling susceptibility to ADEH. Additionally, serum IgE levels might be a surrogate marker for innate immune function and serve as a biomarker for which patients with AD are susceptible to ADEH.
Clinical implications: A deficiency of LL-37 might render patients with AD susceptible to ADEH. Therefore increasing production of skin LL-37 might prevent herpes infection in patients with AD. (J Allergy Clin Immunol 2006;117:836-41.)

Key words: *Antimicrobial peptides, herpes simplex virus, atopic dermatitis, eczema herpeticum*

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects approximately 17% of children and is associated with recurrent skin infections.[1,2] Recent studies have shown that the innate immune response and, more specifically, antimicrobial peptides (AMPs) are decreased in the skin of patients with AD. It has been postulated that this might explain the susceptibility of these individuals to recurrent bacterial skin infections.[3,4]

Eczema herpeticum (ADEH) is a disseminated herpes simplex virus (HSV) 1 or 2 infection that occurs in a subset of patients with AD.[5,6] Left untreated, ADEH might be fatal because of systemic viremia.[7] However, only a small subset of patients with AD have problems with recurrent viral infections, suggesting they might have an AMP phenotype distinct from that of most patients with AD. Therefore this study was conducted to investigate the role of human cathelicidin (LL-37) in controlling HSV infection and to examine differences in cathelicidin expression between patients with uncomplicated AD and patients with ADEH.

From [a]the Division of Allergy and Immunology, Department of Pediatrics, National Jewish Medical and Research Center, and the Department of Pediatrics, University of Colorado Health Sciences Center, Denver; [b]the Department of Dermatology and Allergy, Ludwig Maximilian's University, Munich; [c]the Division of Dermatology, Department of Medicine and Pediatrics, University of California at San Diego and VA San Diego Health Care System.

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Reprint requests: Donald Y. M. Leung, MD, PhD, National Jewish Medical and Research Center, 1400 Jackson St, Room K926i, Denver, CO 80206 E-mail: leungd@njc.org.
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Abbreviations used
AD: Atopic dermatitis
ADEH: Eczema herpeticum
AMP: Antimicrobial peptide
CRAMP: Mouse cathelicidin
HSV: Herpes simplex virus
LL-37: Human cathelicidin
MEM: Minimal essential medium
MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide
pfu: Plaque-forming units

METHODS

Patients

Study participants included 10 patients with AD without a history of ADEH (mean age, 29.3 ± 6.9 years) and 10 patients with AD with a history of ADEH (mean age, 39.8 ± 4.2 years). Patients were classified as having ADEH on the basis of clinical signs of ADEH, as diagnosed by a dermatologist, and a confirmation of HSV infection by means of either PCR or serology. Total serum IgE levels were measured with the UniCAP system (Pharmacia, Uppsala, Sweden). Patients in these studies were never taking oral steroids or systemic calcineurin inhibitors and stopped taking topical calcineurin inhibitors for a minimum of 1 week before enrollment. These studies were conducted according to the Declaration of Helsinki Guidelines and approved by the institutional review board at National Jewish Medical and Research Center in Denver and Ludwig Maximilian's University in Munich. All patients provided written informed consent before participation in these studies.

Skin biopsy specimens were collected from the lesional eczematoid skin rash of patients with AD and ADEH. After collection, skin biopsy specimens were fixed in formalin and archived.

Mice

BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Me). $Cnlp^{-/-}$ (Cnlp knockout) mice were obtained from R. L. Gallo (Veterans Affairs Medical Center and the University of San Diego, San Diego, Calif) and backcrossed onto the BALB/c background. All protocols with these animals were approved by the Institutional Animal Care and Use Committee at National Jewish Medical and Research Center. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection and Research Risks.

Preparation of virus

Human herpes virus type 2 (HSV-2; a gift from Dr Adriana Weinberg, University of Colorado Health Science Center) was grown and passaged in human embryonic lung fibroblasts in Earle's minimal essential medium (MEM; GIBCO, Grand Island, NY) with 2.5% FCS (Gemini Bio Products, Woodland, Calif) and antibiotics. Freshly trypsinized lung fibroblasts were grown for 3 days to confluence and inoculated with approximately 1 plaque-forming unit (pfu) per cell in culture medium. Cells were checked daily for cytopathic effects. The culture supernatant was harvested after 48 to 72 hours of incubation at 37°C in 5% CO_2, freeze-thawed 5 times, and centrifuged for 15 minutes at 1000 rpm. For virus titration, 10-fold dilutions of stock were made, and 0.1 mL of each dilution was added to the fibroblast cell sheets in 24-well tissue-culture plates. Adsorption was allowed to take place for 1 hour at 37°C in 5% CO_2 and was followed by the addition of Earle's MEM with 2.5% FCS. Forty-eight hours after infection, medium was removed, and cells were fixed with formalin-crystal violet. Plaques were visualized on an Inverted Nikon Microscope (Nikon, Tokyo, Japan) under 1.3 × 10 magnification. Virus stocks were stored at −70°C.

Peptide preparations

Human cathelicidin (LL-37) and an irrelevant control peptide, 8044 (GLNGPDIYKGUYQFKSVEFD), were synthesized by means of solid-phase t-BOC chemistry with standard methodology and purified to homogeneity through highly purified liquid chromatography by the Molecular Resource Center at National Jewish. Peptide 8044 was chosen from a library of existing peptides for use as a control having no sequence identity with the test peptide. The identity of LL-37 was confirmed by means of mass spectroscopy. Concentrations of LL-37 and control peptides used in these experiments ranged from 0 to 100 μM.

Viral killing assay

BS-C-1 African green monkey kidney cells were seeded at 2×10^5 cells/well in 24-well plates (Becton Dickinson, Torreyana, Calif) and allowed to grow to confluence overnight at 37°C in 5% CO_2 in Earle's MEM with 10% FCS and antibiotics. To examine the effects of LL-37 and control peptide, 0 to 100 μM was incubated with 2×10^3 pfu HSV-2 for 24 hours at 37°C in a volume not to exceed 0.1 mL. Growth medium was removed from the cell sheet and rinsed once with Earle's MEM with 2.5% FCS. The virus-protein complex was added to the cells and adsorbed for 1 hour at 37°C in 5% CO_2. Growth medium was added to 0.5 mL and incubated for 24 hours for RNA analysis of HSV gene expression and 48 hours for plaque development. For the plaque assay, the medium was removed, and wells were overlaid with 0.5 mL of 4% buffered formalin and allowed to fix for 10 minutes at room temperature. The formalin was removed, and 0.5 mL of 0.1% crystal violet in PBS was added to the wells for 5 minutes at room temperature. Wells were then aspirated and air-dried for visualization of plaques. We found the most accurate results with the virus alone forming 50 to 80 plaques per well.

Keratinocyte cell culture

HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's medium (Cellgro, Herdon, Va) supplemented with 10% FCS (Gemini Bio Products) and 1% of the following until confluent: penicillin-streptomycin, l-glutamine, MEM with nonessential amino acids (GIBCO), and MEM vitamins solution (GIBCO).

HaCaT cells were infected with 0.05 pfu/cell of HSV-2 for 6 hours to evaluate the antiviral activity of LL-37. After the incubation, HSV-2 was removed, and cells were washed with medium to remove remaining HSV-2. LL-37 (0-100 μM) was added to the cells and allowed to incubate for an additional 18 hours. RNA was isolated from the cells for analysis of HSV-2 gene expression.

Murine skin explant cultures

The dorsal thorax of all mice was clipped and treated with the depilatory agent Nair to remove hair. Seventy-two hours after hair removal, mice were killed by means of CO_2 asphyxiation. Six-millimeter punch biopsy specimens were collected from the dorsal thorax and immediately placed in a 96-well plate and RPMI (Cellgro) supplemented with 10% FCS (Gemini Bio Products). Murine skin biopsy specimens were cultured in the presence of media alone or 2×10^4 pfu HSV-2 for 24 hours. After the exposure period, medium was removed, and biopsy specimens were submerged in Tri-Reagent (Molecular Research Center Inc, Cincinnati, Ohio) for RNA isolation. Three independent experiments were conducted, with a total of 15 mice in each exposure group. Data from one representative experiment are shown.

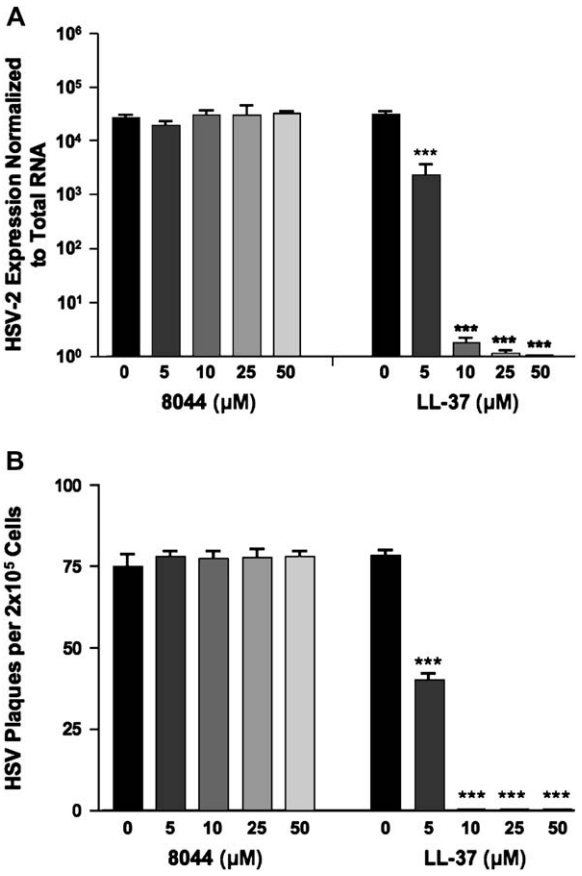

FIG 1. LL-37 exhibits antiviral activity against HSV. Physiologic concentrations of LL-37 were preincubated with HSV-2 for 24 hours and then added to BS-C-1 for an additional 24 hours to evaluate HSV-2 gene expression by means of real-time RT-PCR **(A)** or 48 hours to investigate functionally active virus by using a standard plaque assay **(B)**. ***Significant difference of $P < .001$ compared with 0 μM.

Real-time RT-PCR

Total RNA was isolated from skin biopsy specimens by means of chloroform-phenol extraction and isopropanol precipitation, according to the manufacturer's guidelines (Molecular Research Center Inc). RNeasy Mini Kits (Qiagen, Valencia, Calif) were used, according to the manufacturer's protocol, to isolate RNA from cell cultures and to further purify RNA from skin biopsy specimens. Real-time RT-PCR was performed with an ABI 7000 Sequence Detection system (Applied Biosystems, Foster City, Calif), as previously described.[4] Human GAPDH and rodent GAPDH were purchased from Applied Biosystems. Primer and probe sequences for cathelicidin were designed as previously described.[3] Primer and probe sequences used to assay HSV-2 gene transcripts were as follows: forward, 5′- CGC TCT CGT AAA TGC TTC CCT -3′; reverse, 5′- TCT ACC CAC AAC AGA CCC ACG -3′. This region of the genome encodes glycoprotein D of HSV-2.[8] Relative expression levels were calculated by using the relative standard curve method, as outlined in the manufacturer's technical bulletin. Quantities of all targets in test samples were normalized to the corresponding GAPDH or total RNA levels and expressed as target gene normalized to GAPDH or target gene normalized to total RNA to allow for comparisons between samples and groups. A standard curve was generated with cDNA from purified herpes virus.

Cathelicidin protein expression

Paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, rehydrated, and then stained with rabbit anti-LL-37 (5 μg/mL), as previously described.[3] All slides were coded to ensure patient anonymity, and readings were done blind so that the slide reader was unaware of the identity of the slides. Images were collected at 40× magnification, and the intensity of the immunostaining was scored on a scale from 0 to 5, with 0 indicating no staining and 5 indicating the most intense staining.

Statistical analyses

All statistical analysis was conducted with GraphPad Prism, version 3.01 (San Diego, Calif). Statistical differences in gene expression or protein staining between multiple groups was determined by using a 1-way ANOVA, and significant differences were determined by using the Tukey-Kramer test.[9] Statistical differences in total serum IgE levels were determined by using a Student *t* test.

RESULTS

Anti-HSV activity of LL-37

In our initial experiments we examined whether LL-37 (0-100 μM) could directly kill HSV. As shown in Fig 1, *A*, we observed a concentration-dependent inhibition of viral replication measured by means of real-time RT-PCR. Significant reduction in viral replication by LL-37 was observed with concentrations as low as 5 μM (mean, 2444 ± 1223 ng HSV/ng total RNA; $P < .001$) compared with HSV alone (mean, 32,620 ± 4061 ng HSV/ng total RNA). This was confirmed by using a standard viral plaque assay in which preincubation of HSV with 5 μM of LL-37 significantly ($P < .001$) reduced the number of plaques from 78.3 ± 1.5 (HSV alone) to 40.3 ± 1.9 (Fig 1, *B*). The control peptide 8044 possessed no antiviral activity against HSV.

Role of cathelicidins in controlling HSV replication in the skin

To examine a more physiologic condition, human keratinocyte cultures were preinfected with HSV for 6 hours and then treated with exogenous LL-37 to determine whether intracellular viral replication could be halted with physiologic concentrations of LL-37. Fig 2 demonstrates that concentrations of LL-37 as low as 25 μM (mean, 563 ± 71 ng HSV/ng GAPDH) were able to significantly ($P < .01$) reduce the levels of HSV gene expression in previously infected keratinocytes (HSV mean, 1614 ± 158).

To demonstrate the clinical relevance of LL-37 compared with other potential arms of the innate immune response in limiting HSV infection, we used mice deficient in Cnlp, the murine cathelicidin. Significantly higher levels of HSV replication were observed in skin biopsy specimens from Cnlp knockout mice (BALB/c background; 2.6 ± 0.5 pg HSV/pg GAPDH, $P < .05$) compared with that seen in skin biopsy specimens from wild-type BALB/c mice (0.9 ± 0.3 pg HSV/pg GAPDH, Fig 3), suggesting that cathelicidins play an

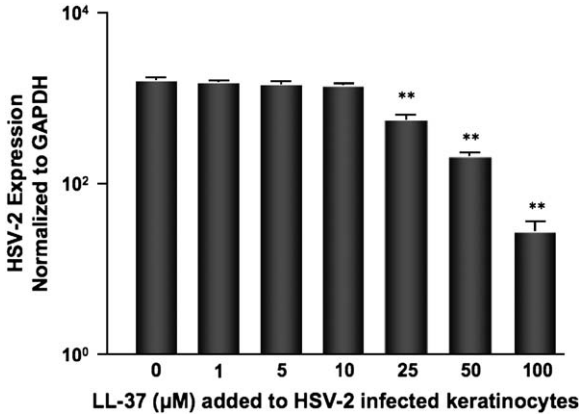

FIG 2. Exogenous LL-37 rescues HSV-infected keratinocytes. Human keratinocytes were infected with 0.05 pfu/cell HSV-2 for 6 hours and then treated with physiologic concentrations of LL-37 for an additional 18 hours. RNA was isolated from the cells, and the levels of HSV-2 gene expression were evaluated by means of real-time RT-PCR. **Significant difference of $P < .01$ compared with HSV alone.

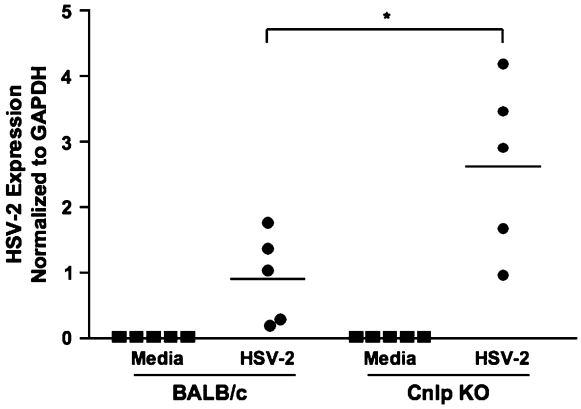

FIG 3. Essential role of cathelicidins in controlling HSV replication in the skin. Skin biopsy specimens from BALB/c (n = 5) and Cnlp knockout (n = 5) mice were stimulated with HSV-2 for 24 hours and evaluated for HSV-2 gene expression. RNA was collected from the tissue, and the levels of HSV-2 were evaluated by means of real-time RT-PCR. *Significant difference of $P < .05$.

important role in controlling HSV skin infection. These data are representative of 3 independent experiments with a total of 15 mice.

Deficiency of LL-37 in the skin of patients with ADEH

Skin biopsy specimens were collected from the skin lesions of adult patients with AD and patients with ADEH. Biopsy specimens were stained with a polyclonal antibody specific for LL-37 to investigate cathelicidin expression. All slides were coded before analysis, and readings were done blind so that the slide reader was unaware of the identity of the slides. Fig 4, *A*, shows that skin sections from patients with AD exhibited more staining for cathelicidin than skin lesions from patients with ADEH. The composite data for cathelicidin immunostaining in all samples is shown in Fig 4, *B*. The intensity of cathelicidin staining in ADEH skin lesions was significantly ($P < 0.05$) lower than that seen in skin lesions from patients with AD.

Correlation between serum IgE level and cathelicidin expression

Previous studies have suggested that T_H2 cytokines could inhibit cathelicidin production.[10] Because of the limited amount of archived tissue available, we were unable to investigate potential differences in IL-4 and IL-13 expression between patients with AD and patients with ADEH. However, IL-4 and IL-13 are T_H2 cytokines essential in the production of IgE.[11] Therefore we examined whether increases in serum IgE levels might correlate with cathelicidin expression in patients with ADEH and patients with AD. Because antibody generation is exponential, we log transformed the serum IgE values for further statistical analysis. Using linear regression analysis, we demonstrate a significant correlation ($r^2 = 0.46$, $P < .05$) between total serum IgE levels and cathelicidin protein expression in patients with AD and patients with ADEH (Fig 5).

DISCUSSION

AMPs are an integral part of the innate immune response because they have been shown to be effective in killing bacterial[3] and viral[12] pathogens. Cathelicidin is produced by several cells in the skin, including keratinocytes, where it is induced in response to inflammatory stimuli.[13,14] On release, the cathelicidin precursor protein is processed into the biologically active AMP LL-37. In this study we demonstrate that LL-37 exhibits antiviral activity against HSV. Our previous studies have demonstrated that patients with AD, in general, have a reduced ability to generate cathelicidin in their skin compared with patients with psoriasis or allergic contact dermatitis, and this might predispose them to microbial skin infection.[3,10] However, the propensity of patients with AD to have serious skin infection, such as ADEH, has not previously been explored. Results from the current study indicate that a more exaggerated reduction in cathelicidin expression might predispose a subset of patients with AD to having ADEH. Thus there is heterogeneity in the expression of cathelicidin within AD, such that the individuals with the lowest levels are most prone to disseminated viral infection.

It was previously reported that LL-37 exhibits little activity against HSV-1 or HSV-2.[15] In contrast, we demonstrate in this study that LL-37 exhibits potent antiviral activity against HSV. In the previous study Yasin et al[15] demonstrated that 44.5 μM of LL-37 provided 28% and 46% protection against HSV-1 and HSV-2, respectively, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. In the MTT assay antiviral activity of LL-37 is determined on the basis

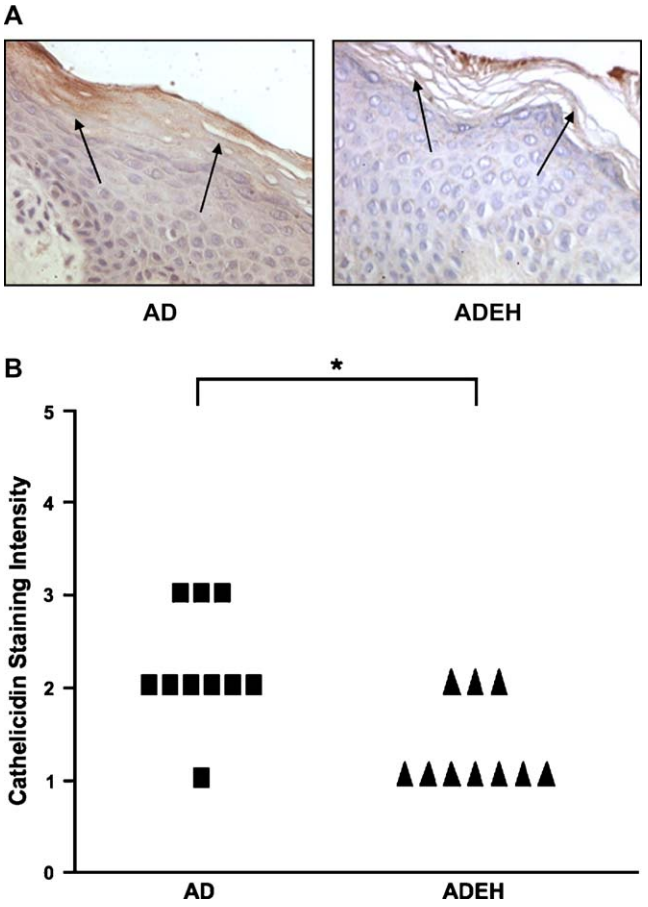

FIG 4. Expression of cathelicidin increased in AD skin compared with that seen in ADEH skin. **A**, Paraffin-embedded skin explants from patients with AD (n = 10) and patients with ADEH (n = 10) were cut into 5-μm sections and stained for human cathelicidin. **B**, The intensity of the immunostaining was visually scored on a scale from 0 to 5, with 0 indicating no staining and 5 indicating the most intense staining. *Significant difference of $P < .05$.

of increases in cellular proliferation compared with cells treated with virus alone. In our current study we measured HSV replication by evaluating glycoprotein D gene expression using real-time RT-PCR. Directly measuring the virus activity rather than cellular proliferation provides a more direct and appropriate measurement of the antiviral activity of LL-37 against HSV, accounting for differences between our results and those of Yasin et al.[15] Our observation is further supported by Gordon et al,[16] who recently demonstrated that LL-37 exhibits antiviral activity against HSV-1 in corneal and conjunctival epithelia.

In this study we demonstrate that concentrations as low as 10 μM of LL-37 reduce HSV levels by more than 10,000-fold. Because psoriatic skin can contain up to 1605 μM LL-37,[3] this demonstrates that physiologic concentrations of LL-37 are effective at controlling HSV replication. This was further supported by using a more physiologic approach in which keratinocytes were preinfected with HSV for 6 hours and then incubated with LL-37 for an additional 18 hours. Again, we saw greater than a 60% reduction in the levels of HSV when as little as 25 μM of LL-37 was added to previously infected keratinocytes. The importance of cathelicidins in skin innate immune responses to HSV is also strongly supported by our current finding that skin explants from mice deficient in the cathelicidin gene Cnlp and its AMP product, mouse cathelicidin (CRAMP), sustain higher levels of HSV replication after inoculation compared with those seen in their wild-type counterparts. Mouse CRAMP is very similar to human LL-37 in structure, tissue distribution, and antimicrobial activity and is therefore a reasonable model of the human cathelicidin. The observation that CRAMP-deficient mice support a higher level of HSV replication reinforces the important effect that a cathelicidin deficiency would have on HSV skin infection in human subjects. These data support a conclusion that decreased cathelicidin expression will significantly increase the potential for disseminated skin infection to occur.

Because not all patients with AD have ADEH, we investigated the abundance of cathelicidin in the skin of patients with AD and patients with ADEH to determine whether the development of ADEH corresponded with decreased cathelicidin expression. Skin biopsy specimens were obtained from naturally induced inflammatory skin rashes of AD and ADEH. This allowed for comparisons

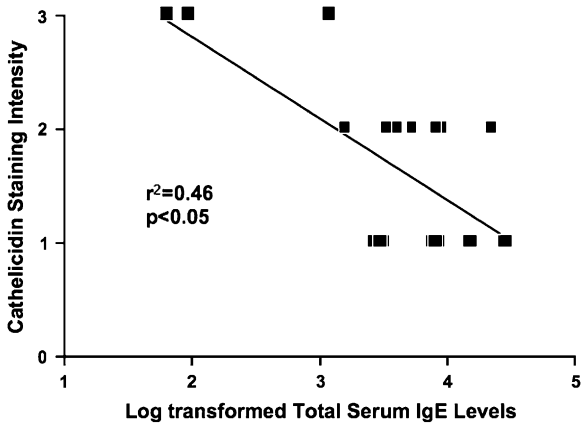

FIG 5. Correlation between serum IgE levels and cathelicidin expression in patients with AD and patients with ADEH. Serum IgE levels were determined from patients with AD (n = 9) and patients with ADEH (n = 9). Regression analysis was performed on log-transformed serum IgE values and LL-37 protein expression.

between similarly stimulated skin samples. Lesional skin from patients with ADEH exhibited significantly lower levels of cathelicidin protein than skin lesions of patients with uncomplicated AD. Our results suggest that patients with AD with the lowest levels of cathelicidin are most susceptible to development of ADEH and that lack of this molecule might serve as a biomarker for patients at risk for disseminated viral skin infection.

AD skin is characterized by the overexpression of the T_H2 cytokines IL-4 and IL-13.[17] Previously, we demonstrated that T_H2 cytokines downregulate cathelicidin.[10] However, because of the difficulty in identifying patients with ADEH and insufficient amounts of archived tissue, we were unable to investigate the levels of IL-4 and IL-13 in patients with AD and patients with ADEH for potential differences. IL-4 and IL-13 are T_H2 cytokines essential in the production of IgE.[11] Therefore the measurement of serum IgE levels might serve as a biomarker for levels of T_H2 responses. Wollenberg et al[6] and Lagace-Simard et al[18] have previously demonstrated that patients with ADEH exhibited higher total serum IgE levels than patients with AD. We confirmed this observation in the current study by demonstrating significantly higher serum IgE levels in patients with AD compared with those seen in patients with ADEH. We further determined that there is a strong correlation between the levels of serum IgE and cathelicidin protein expression in patients with AD and patients with ADEH. Therefore serum IgE levels might serve as a surrogate marker for the expression of cathelicidin in the skin of patients with AD and patients with ADEH and might separate those who are more susceptible to disseminated viral infection.

The current study therefore demonstrates that AD represents a heterogeneous population of patients expressing different levels of cathelicidin in the skin. This explains, in part, why a subgroup of patients with AD is susceptible to ADEH after HSV infection. Additionally, this study demonstrates the importance of the cathelicidin in controlling the replication of HSV in the skin. This is supported by significantly higher levels of HSV replication in the skin of Cnlp knockout mice and the significant reduction of HSV gene expression in keratinocytes treated with LL-37. Overall, these data suggest further clinical studies are warranted to examine whether augmentation of LL-37 expression in AD skin might be useful in the prevention of ADEH and the perplexing challenge of controlling microbial infection in this common skin problem.

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