

ENDOGENOUS ANTIMICROBIAL PEPTIDES AND SKIN INFECTIONS IN ATOPIC DERMATITIS

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ABSTRACT

Background The innate immune system of human skin contains antimicrobial peptides known as cathelicidins (LL-37) and β -defensins. In normal skin these peptides are negligible, but they accumulate in skin affected by inflammatory diseases such as psoriasis. We compared the levels of expression of LL-37 and human β -defensin 2 (HBD-2) in inflamed skin from patients with atopic dermatitis and from those with psoriasis.

Methods The expression of LL-37 and HBD-2 protein in skin-biopsy specimens from patients with psoriasis, patients with atopic dermatitis, and normal subjects was determined by immunohistochemical analysis. The amount of antimicrobial peptides in extracts of skin samples was also analyzed by immunodot blot analysis (for LL-37) and Western blot analysis (for HBD-2). Quantitative, real-time reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays were used to determine the relative expression of HBD-2 and LL-37 messenger RNA (mRNA) in the skin-biopsy specimens. These peptides were also tested for antimicrobial activity against *Staphylococcus aureus* with the use of a colony-forming assay.

Results Immunohistochemical analysis confirmed the presence of abundant LL-37 and HBD-2 in the superficial epidermis of all patients with psoriasis. In comparison, immunostaining for these peptides was significantly decreased in acute and chronic lesions from patients with atopic dermatitis ($P=0.006$ and $P=0.03$, respectively). These results were confirmed by immunodot blot and Western blot analyses. Real-time RT-PCR showed significantly lower expression of HBD-2 mRNA and LL-37 mRNA in atopic lesions than in psoriatic lesions ($P=0.009$ and $P=0.02$, respectively). The combination of LL-37 and HBD-2 showed synergistic antimicrobial activity by effectively killing *S. aureus*.

Conclusions A deficiency in the expression of antimicrobial peptides may account for the susceptibility of patients with atopic dermatitis to skin infection with *S. aureus*. (N Engl J Med 2002;347:1151-60.)

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THE skin's first line of defense against invasion by microbial agents is the stratum corneum, a nonviable, desiccated layer of the epidermis.¹ However, this physical barrier is susceptible to injuries that allow the entry of opportunistic microbial agents into the skin. The innate immune system can immediately respond to this intrusion by helping to prevent further invasion. This immune response includes phagocytosis by neutrophils and macrophages and their production of reactive oxygen intermediates that kill microbial agents.²

A number of endogenous antimicrobial peptides have been shown to play an integral part in innate immunity.³ Two major classes of peptides in mammalian skin, β -defensins^{4,5} and cathelicidins,^{6,7} have antimicrobial activity against bacterial, fungal, and viral pathogens.^{4,7,9} These peptides, which are produced by keratinocytes in the skin,^{4,7,8} disrupt the membrane of the target microbe or penetrate the microbial membrane, interfering with intracellular functions.¹⁰ The expression of some of these peptides, such as human β -defensin 1 (HBD-1), is constitutive,⁸ whereas the expression of others, including human β -defensin 2 (HBD-2) and LL-37, a cathelicidin, is triggered by injury or inflammation of the skin.^{4,7} Animal models have shown that the expression or activation of antimicrobial peptides is essential for the ability of skin to resist bacterial infection.^{11,12}

Atopic dermatitis, a chronic inflammatory skin disease frequently found in families with asthma and allergic rhinitis,¹³ is complicated by recurrent infections of skin lesions by bacterial, viral, and fungal pathogens.¹⁴ About 30 percent of patients with atopic dermatitis have bacterial or viral infections of the skin, as compared with only 7 percent of patients with psoriasis,¹⁵ even though both diseases are characterized by a defective skin barrier.¹⁶ We compared the expression of HBD-2 and LL-37 in skin lesions from pa-

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tients with atopic dermatitis with their expression in psoriatic lesions and normal skin, using immunohistochemical staining, Western and immunodot blotting, and a quantitative, real-time reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay. We also examined the capacity of tumor necrosis factor α (TNF- α) to induce HBD-2 expression in a human keratinocyte cell line after treatment with interleukin-4 and interleukin-13, cytokines that are abundant in the skin of patients with atopic dermatitis.¹³ Finally, we evaluated the combined antimicrobial effects of LL-37 and HBD-2 on *Staphylococcus aureus*.

METHODS

Patients

The study participants included 8 patients with moderate-to-severe atopic dermatitis (mean age, 33 years; extent of skin involvement, 20 to 60 percent), 11 patients with psoriasis (mean age, 38 years; extent of skin involvement, 15 to 40 percent), and 6 healthy persons (mean age, 38 years). None of the patients had received systemic corticosteroids previously, and none had received topical corticosteroids for at least one week before enrollment. The study was approved by the institutional review board at National Jewish Medical and Research Center, in Denver; all patients and normal subjects gave written informed consent.

Punch biopsies were performed, with 2-mm samples obtained from erythematous lesions that were less than three days old (acute atopic dermatitis), lichenified lesions that were more than two weeks old (chronic atopic dermatitis), psoriatic lesions, and normal skin. The skin samples were immediately frozen at -70°C for immunohistochemical studies or Western and immunodot blot analyses.

Immunohistochemical Staining

For immunostaining of HBD-2, frozen 5- μm skin-tissue sections were fixed in acetone for 10 minutes and then incubated with 10 percent nonimmune goat serum (Zymed) for 10 minutes. Blocking serum was removed, and the sections were stained with anti-HBD-2 rabbit polyclonal antibody (Peptide Institute) at a 1:50 dilution (vol/vol) in phosphate-buffered saline for one hour at room temperature in a humid atmosphere. The slides were rinsed twice with phosphate-buffered saline and incubated with a biotinylated goat antirabbit secondary antibody (Zymed) at room temperature for 30 minutes, rinsed twice with phosphate-buffered saline, and incubated with a mixture of avidin and biotinylated horseradish peroxidase (1:1 vol/vol, Dako) for an additional 30 minutes. The reaction was developed with the use of amino-ethyl-carbazole single solution (Zymed) for 10 minutes and then counterstained with hematoxylin. Negative controls were established with the use of non-immune isotype antibodies and preincubation of the anti-HBD-2 antibodies with a synthetic HBD-2 peptide (Peptide Institute) to ensure the binding specificity of the anti-HBD-2 antibodies. All slides were coded before the samples were evaluated so that the identity of the study subjects was not revealed. The intensity of the immunostaining was graded with the use of microscopy on a scale from 0 to 3, with 0 indicating no staining and 3 the most intense staining.

For immunostaining of LL-37, frozen skin sections were treated similarly at first and then incubated with rabbit anti-LL-37 antibody, as previously described,¹⁷ in phosphate-buffered saline and 0.1 percent bovine serum albumin. The sections were washed in phosphate-buffered saline and stained with goat antirabbit horseradish peroxidase (Vectastain Elite ABC Rabbit kit, Vector Laboratories) and diaminobenzidine substrate (Sigma) according to the manufacturer's instructions. The sections were counterstained with

hematoxylin. The specificity of the primary antibody reaction was confirmed in separate experiments by adsorption of either anti-LL-37 antibody with excess amounts of the synthetic peptide. The specificity of the secondary antibody reaction and of the immunostaining reagents was confirmed by routine use of rabbit non-immune serum.

Measurement of HBD-2 and LL-37 Peptides

Skin-biopsy samples were weighed and then homogenized for 10 minutes with a loose-fitting Dounce homogenizer in 1 ml of 1 M hydrogen chloride and 1 percent trifluoroacetic acid on ice. Homogenized tissues in solution were then rotated overnight at 4°C . After centrifugation for 20 minutes at 14,000 rpm at 4°C , supernatants were transferred to new tubes and lyophilized completely. The resulting protein pellets were dissolved with distilled water to obtain a final protein concentration of 0.1 mg of tissue per microliter.

For the measurement of HBD-2, 15 μl of each sample was lyophilized again and resuspended in sodium dodecyl sulfate buffer overnight at 4°C . Samples and known amounts of HBD-2 for use as standards were then boiled for five minutes and loaded onto a 16.5 percent sodium dodecyl sulfate-tricine polyacrylamide gel. The samples were placed on Immobilon-PSQ membranes (Millipore) for one hour at 0.18 mA in 0.05 M sodium borate, pH 9.0, with 20 percent methanol and 0.05 percent sodium dodecyl sulfate. Blots were fixed for 30 minutes with 0.5 percent glutaraldehyde in TRIS-buffered saline (500 mM sodium chloride and 20 mM TRIS, pH 7.5), blocked for 30 minutes in 0.75 percent Blotto (nonfat powdered milk) in phosphate-buffered saline (0.9 percent sodium chloride and 10 mM sodium phosphate buffer, pH 7.4), then incubated for 18 hours in a 1:500 dilution of rabbit anti-HBD-2 serum in antibody dilution buffer (0.25 percent Blotto in phosphate-buffered saline containing 0.01 percent thimerosal as a preservative). The blots were washed in 0.1 percent bovine serum albumin in Blotto TRIS-buffered saline (0.9 percent sodium chloride and 20 mM TRIS-hydrogen chloride, pH 4.5 to 5.0) three times for 10 minutes each time. The membranes were incubated in a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG in antibody dilution buffer for one hour, then washed three times as before and developed in alkaline phosphatase development solution (bromochloroindolyl phosphate-nitroblue tetrazolium).

For the measurement of LL-37, immunodot blot analysis was performed. Positive controls and standard curves were generated with a synthetic LL-37 peptide (C-terminal, 18-mer fragment; amino acid sequence, CZQPIKDFLRNLPRTES; molecular weight, 2204). The peptide was diluted serially from 50 to 1600 nM; 100 μl of each peptide standard and 5 μl of each sample (equivalent to 0.5 mg of tissue), extracted in a manner identical to that for HBD-2 measurements, were dotted in triplicate on nitrocellulose membrane. The membrane was blocked with 10 percent nonfat dry milk (Bio-Rad) and 0.1 percent Tween 20 in TRIS-buffered saline at room temperature for three hours and then incubated at 4°C overnight with rabbit anti-LL-37 antibody diluted to 1:2500 in the blocking solution. The membrane was then incubated at room temperature for two hours with goat antirabbit antibody-horseradish peroxidase (Dako) that was diluted to 1:4000 in blocking solution. Western Lightning chemiluminescence reagent (Perkin-Elmer Life Sciences) was used to detect electro-chemiluminescence. A low-light imaging system (ChemiImager 4400, Alpha Innotech) was used for densitometrical analysis. Signal detected by dot blot analysis was confirmed in some experiments by Western blot analysis, which identified full-length LL-37 at 18 kD. For quantification of both LL-37 and HBD-2, the signal intensity of tissue extracts was directly compared with that of a simultaneously prepared standard consisting of known amounts of each synthetic peptide. The concentration of antimicrobial peptide in the original skin-biopsy sample was then estimated by dividing the experimentally determined mass of antimicrobial peptide from the skin-biopsy

sample by the epidermal volume. The epidermal volume was estimated by assuming an epidermal thickness of 0.1 mm; thus, the volume of epidermis in a 2-mm punch-biopsy specimen was $3.14 \times 10^{-1} \text{ mm}^3$.

RT-PCR Analysis of HBD-2 and LL-37 Messenger RNA

Total RNA was isolated from 2-mm skin-biopsy samples from the subjects with the use of TRI Reagent (Sigma) according to the manufacturer's protocol. The primers and probe for HBD-2 were designed with the use of Prism 7700 sequence-detection software (Primer Express, Perkin-Elmer Applied Biosystems). The primer sequences were 5'TCCTCTTCTCGTTCCTCTTCATATTC3' (forward primer) and 5'TTAAGGCAGGTAACAGGATCGC3' (reverse primer). The sequence for the probe (TaqMan, Perkin-Elmer) was 5'ACCACAAAACACCTGGAAGAGGCA3'; the 5' end was labeled with 6-carboxyfluorescein, and the 3' end was labeled with 6-carboxytetramethylrhodamine.

Amplification reactions were performed with the use of a sequence detector (ABI Prism 7700, Perkin-Elmer Applied Biosystems) in MicroAmp optical tubes (Perkin-Elmer Applied Biosystems) in a 25- μl mixture containing 8 percent glycerol, $1 \times$ TaqMan buffer A (500 mM potassium chloride, 100 mM TRIS-hydrogen chloride, 0.1 M EDTA, and 600 nM passive reference dye ROX, pH 8.3, at room temperature), 300 μM each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate, and 600 μM deoxyuridine triphosphate; 5.5 mM magnesium chloride; 900 nM forward primer; 900 nM reverse primer; 200 nM probe; 0.625 U of AmpliTaq Gold DNA Polymerase (Perkin-Elmer); 6.25 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies); 10 U of RNasin ribonuclease inhibitor (Promega); and the template RNA. The RT assay was performed at 48°C for 30 minutes, followed by activation of TaqGold at 95°C for 10 minutes. Forty cycles of amplification were then performed at 95°C for 15 seconds and at 60°C for 1 minute.

After amplification, real-time data acquisition and analysis were performed. The fluorescence data were expressed as normalized reporter signal, calculated by dividing the amount of reporter signal by the amount of passive reference signal, or as the change in the normal reporter signal, calculated as the amount of normalized reporter signal minus the amount of reporter signal before PCR. The detection threshold was set above the mean base-line value for fluorescence determined on the basis of the first 15 cycles. Amplification reactions in which the intensity of fluorescence exceeded the threshold were defined as positive reactions. The threshold cycle (Ct) is the PCR cycle at which an increase in reporter signal above the base-line signal can first be detected. A standard curve was generated with the use of the fluorescence data from the 10-fold serial dilutions of total RNA from a skin-biopsy sample from a patient with psoriasis. This curve was used to calculate the relative amounts of HBD-2 in test samples. Quantities of HBD-2 in test samples were normalized to the corresponding 18S ribosomal RNA (rRNA) (P/N 4308310, Perkin-Elmer Applied Biosystems).

The real-time, quantitative PCR assay for LL-37 was performed with the use of a sequence-detection system (GeneAmp 5700, Perkin-Elmer). Classic 18S primers (Ambion) were used to amplify 18S rRNA. The primer sequences used to amplify LL-37 complementary DNA (cDNA) were 5'GCAGTCACCAGAGGATTGTGAC3' (forward primer) and 5'CACCGCTTACCAGCCC3' (reverse primer). For the PCR assay of LL-37, 1.2 μl of RT reaction was used; 1.2 μl of 200-fold diluted RT reaction was used for PCR of 18S rRNA. Amplification reactions were performed in a final total volume of 25.7 μl containing SYBR Green PCR Master Mix (Applied Biosystems), 10 μM primer, and nuclease-free water. The assay was performed at 50°C for 2 minutes and at 95°C for 10 minutes. Forty cycles of amplification were then performed at 94°C for 15 seconds and at 60°C for 1 minute. The results were analyzed with the use of the comparative Ct method. This method is based on the assumption that the target (LL-37) and reference (18S)

primers amplify with the same efficiency within a given range of initial messenger RNA (mRNA) concentrations. To test this assumption, cDNA was made from total RNA of a psoriasis sample at the initial total RNA concentrations of 1000, 500, 100, 50, and 10 ng. Real-time PCR was performed in triplicate for each starting concentration, and the change in Ct (ΔCt) was calculated. For each starting concentration of total RNA, $\Delta\text{Ct} = \text{Ct}_R - \text{Ct}_G$, where Ct_R denotes the reference Ct and Ct_G the target Ct. This value was plotted against the log of the initial total RNA concentration. The efficiencies of gene amplification were close enough that a correction factor over the specified range was not required if the slope was less than or equal to 0.1. We calculated the $\Delta\Delta\text{Ct}$ for samples as follows: $\Delta\Delta\text{Ct} = \Delta\text{Ct}_E - \Delta\text{Ct}_B$, where Ct_E denotes the experimental Ct and Ct_B the base-line Ct. The base-line sample was a normal control sample, which had low expression of LL-37 mRNA. The relative expression was calculated as $2^{\Delta\Delta\text{Ct}}$ in order to account for the exponential amplification of the PCR reaction.

Cell Culture

HaCat cells, a human keratinocyte cell line, were grown in Dulbecco's Modified Eagle's medium (Cellgro), supplemented with 10 percent fetal-calf serum (Gemini) and 1 percent of each of the following: 200 mM L-glutamine, minimal essential medium (MEM) with nonessential amino acids (GIBCO), MEM Vitamins Solution (Life Technologies), and penicillin-streptomycin. To study the effects of interleukin-4 and interleukin-13 on HBD-2 mRNA expression in HaCat cells, 5×10^5 cells per milliliter were incubated in control medium, 20 ng of TNF- α per milliliter alone or 20 ng of TNF- α per milliliter plus 50 ng of interleukin-4 per milliliter, alone or in combination with 50 ng of interleukin-13 per milliliter, for 24 hours. The cells were then washed once and homogenized in TRI reagent by repeated pipetting. Total RNA was isolated according to the manufacturer's protocol.

Measurement of Antimicrobial Activity

LL-37 and HBD-2 peptides were synthesized as previously described.^{17,18} The antimicrobial activity of the peptides was determined with the use of a solution colony-forming unit (CFU) assay. Wild-type *S. aureus* isolated from patients with atopic dermatitis was assessed. Bacteria were grown to a concentration at which they were multiplying exponentially (mid log phase; $A_{600} 1.5 = 8.0 \times 10^9$ CFU per milliliter) in Todd Hewitt broth medium (Sigma). Bacteria were washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 percent (wt/vol) Todd Hewitt broth and were diluted to a final concentration of 2.0×10^7 cells per milliliter in the same buffer. Ten microliters of this bacterial suspension was then placed in round-bottom, 96-well cell-culture plates and incubated at 37°C and 100 percent humidity for two hours with various concentrations of LL-37, HBD-2, or both. The cytotoxic activity and minimal bactericidal activity of LL-37 and HBD-2 were analyzed by plating serial dilutions of the incubation mixture in triplicate on Todd Hewitt broth agar plates and determining the CFU the following day. The percentage of killed bacteria was expressed as $[1 - (\text{CFU after peptide incubation}) \div (\text{CFU after control incubation})] \times 100$.

Statistical Analysis

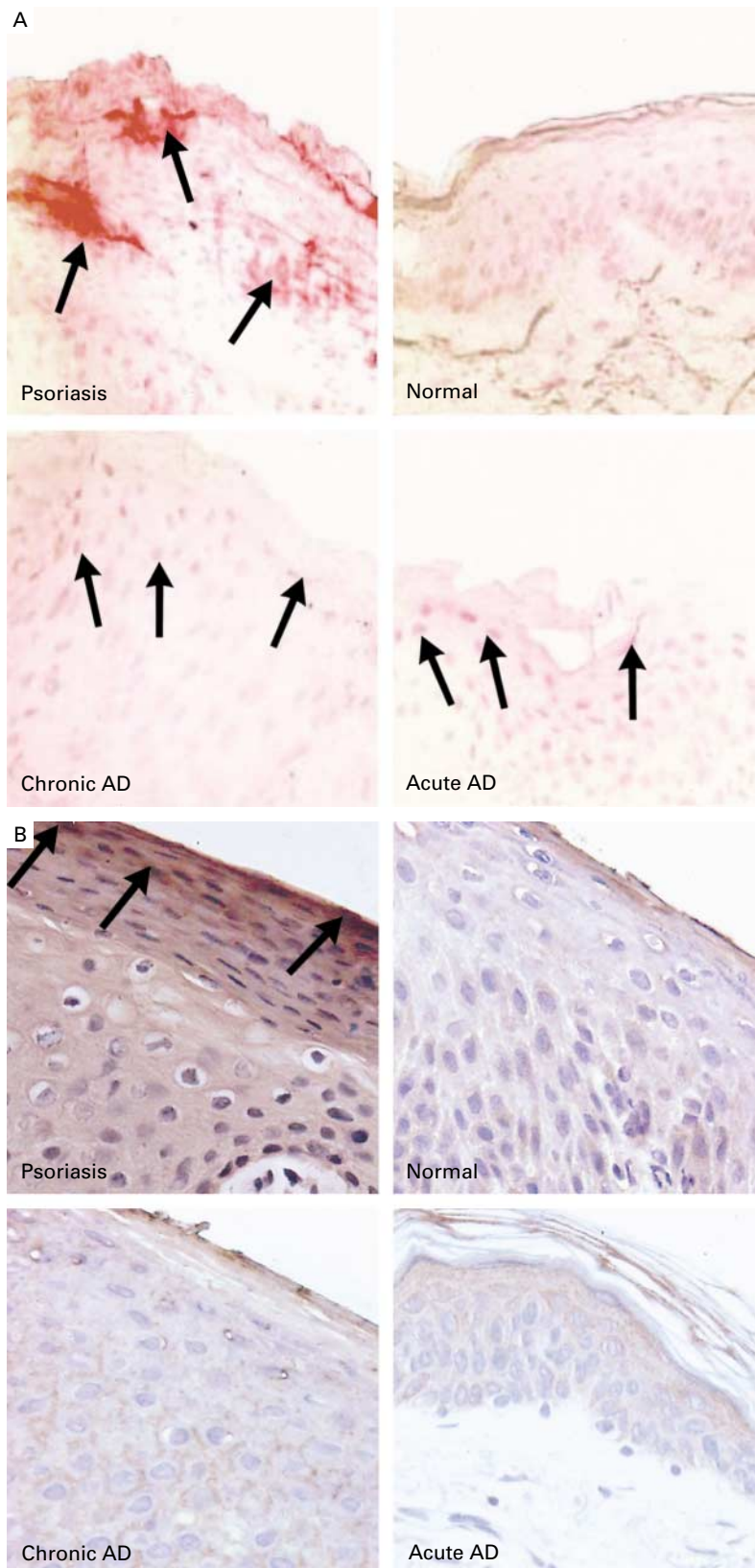
The nonparametric median test from the JMP4 statistical package¹⁹ was used to determine significant differences. We constrained experimentwise error rates to the standard alpha level of 0.05 by the Bonferroni method; the alpha level was 0.025 for a comparison of two means and 0.017 for a comparison of three means.

RESULTS

Figure 1 shows the immunostaining of HBD-2 and LL-37 in skin-biopsy samples. The samples from psoriasis

Figure 1. Immunostaining for HBD-2 (Panel A) and LL-37 (Panel B) in Frozen Skin Sections from Patients with Psoriasis or Atopic Dermatitis (AD) and Normal Subjects.

Immunostaining for HBD-2 and LL-37 was more intense in the psoriatic lesions than in the atopic lesions or normal skin. The arrows in Panels A and B indicate immune reactivity to HBD-2 and LL-37, respectively.



riatic lesions had much more intense staining for both HBD-2 and LL-37 than the samples from acute or chronic atopic lesions or normal skin. Figure 2 shows the composite data on HBD-2 immunostaining for all the skin-biopsy samples. The intensity of the immunostaining of samples from acute atopic lesions did not differ significantly from that of chronic atopic lesions or normal skin. Psoriatic lesions, however, had significantly more intense immunostaining than acute atopic lesions ($P=0.006$), chronic atopic lesions ($P=0.03$), or normal skin ($P=0.02$). The results of immunostaining for LL-37 in the samples of normal skin and psoriatic lesions were consistent with a previous study showing that psoriatic lesions have greater expression of LL-37 than normal skin.⁷ The inflammatory skin lesions from patients with atopic dermatitis had markedly lower expression of LL-37 than the skin lesions from patients with psoriasis. The biggest difference was in the region of the granular cell layer and stratum corneum, which was relatively devoid of LL-37 in the atopic lesions but had the highest expression of LL-37 in the psoriatic lesions. The absence of HBD-2 and LL-37 in the normal skin also confirms previous reports that these antimicrobial peptides are

not normally produced but are up-regulated under inflammatory conditions.^{4,7}

For further quantification of the relative amounts of antimicrobial peptides in skin lesions, 2-mm biopsy specimens were acid-extracted, and the total amount of LL-37 and HBD-2 was measured (Fig. 3). Sufficient skin samples were available for analysis from only five patients with psoriasis and six with atopic dermatitis. Western blotting, as compared with a standard curve of recombinant HBD-2, showed that the amount of HBD-2 present in the psoriatic lesions ranged from 2.3 to 157 μM (median, 20), whereas no HBD-2 was detectable in the atopic lesions (Fig. 3A). The results of quantification of LL-37 in skin-biopsy samples with the use of immunodot blot analysis were similar, with an abundance of LL-37 in the psoriatic lesions (median, 304 μM ; range, 0 to 1605); only one of the six atopic lesions had detectable LL-37 (Fig. 3B). The single positive sample from a patient with atopic dermatitis was from a chronic lesion, probably reflecting the contribution of LL-37 from an inflammatory-cell infiltrate.

The difference in the expression of HBD-2 and LL-37 protein between atopic lesions and psoriatic

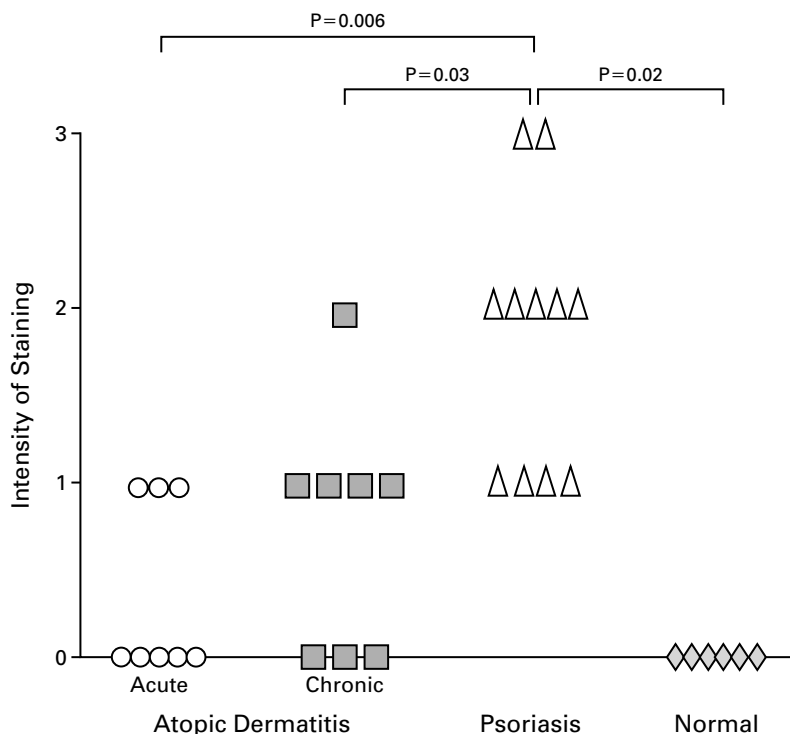


Figure 2. Intensity of Immunostaining for HBD-2 in Skin from Patients with Atopic Dermatitis, Patients with Psoriasis, and Normal Subjects.

The intensity of the staining was graded visually on a scale from 0 (no staining) to 3 (the most intense staining). There was no staining in any of the skin-biopsy samples from normal subjects.

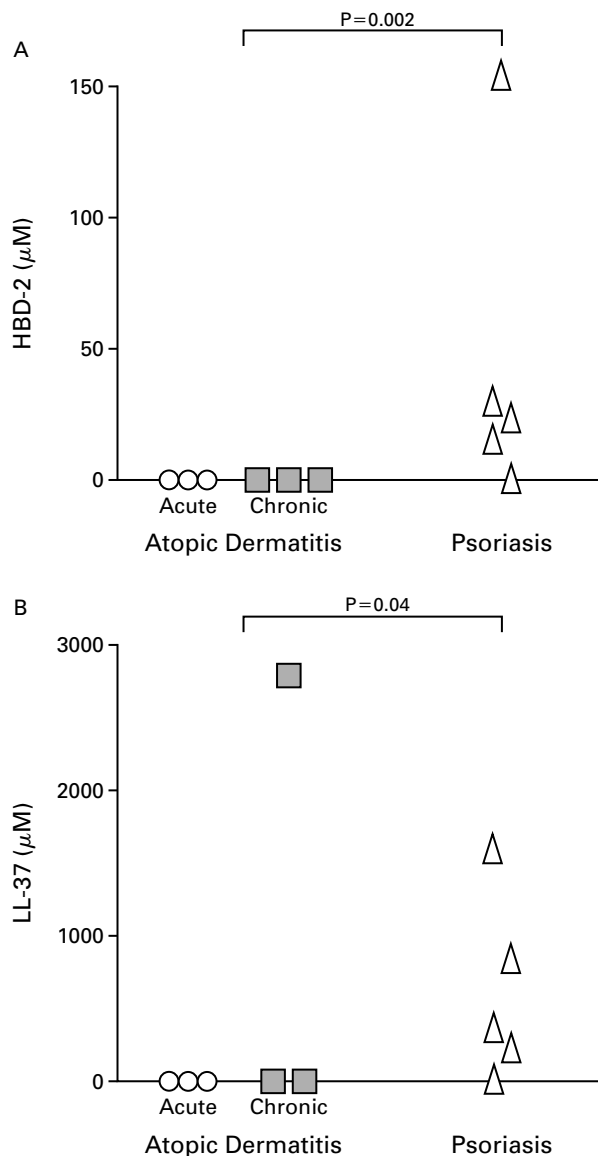


Figure 3. Quantification of HBD-2 (Panel A) and LL-37 (Panel B) in Epidermis of Atopic and Psoriatic Skin Lesions.

Data for HBD-2 are based on Western blot analysis, and data for LL-37 on immunodot blot analysis.

lesions was further confirmed by the measurement of mRNA with the use of real-time RT-PCR. As shown in Figure 4, the expression of HBD-2 mRNA was significantly lower in acute atopic lesions (median, 98 pg per nanogram of rRNA) and chronic atopic lesions (median, 3268 pg per nanogram of rRNA) than in psoriatic lesions (median, 31,660 pg per nanogram of rRNA; $P=0.009$ for both comparisons). Real-time RT-PCR analyses of LL-37 mRNA showed similar results, with significantly lower expression of LL-37 pep-

ptide in acute and chronic atopic lesions than in psoriatic lesions ($P=0.02$ for both comparisons) (Fig. 4).

Previous studies have shown that LL-37 and HBD-2 are greatly increased in patients with inflammatory skin conditions.⁵⁻⁷ Therefore, the diminished expression of the peptides in lesions from patients with atopic dermatitis was an unexpected observation. To determine the mechanism underlying the reduced expression of antimicrobial peptides in atopic dermatitis, we studied the effects of two cytokines (interleukin-4 and interleukin-13) that are elevated in atopic dermatitis but not in psoriasis.¹³ Figure 5 shows the effects of interleukin-4 and interleukin-13 on TNF- α -induced expression of HBD-2 in HaCat cells. Interleukin-4 alone or in combination with interleukin-13 significantly suppressed the up-regulation of HBD-2 mRNA by TNF- α ($P=0.04$). In normal human skin, both interleukin-4 and interleukin-13 suppressed TNF- α -induced HBD-2 mRNA expression in these explants (data not shown). Since atopic lesions are characterized by the predominance of interleukin-4 and interleukin-13 expression, the suppression of HBD-2 mRNA by these cytokines may account for the low expression of HBD-2 in these lesions.

The relative absence of HBD-2 and LL-37 in the epidermis in atopic lesions, as compared with psoriatic lesions, suggested that the lack of these antimicrobial peptides in patients with atopic dermatitis might contribute to their susceptibility to bacterial colonization and infection. To investigate this possibility, we evaluated the susceptibility of clinical isolates of *S. aureus* to cytotoxic activity by both LL-37 and HBD-2. Alone, LL-37 showed strong activity (Fig. 6A), whereas HBD-2 was much less potent (minimal bactericidal concentration, $>160 \mu\text{M}$). No significant difference in susceptibility to these antimicrobial peptides was detected in clinical isolates, as compared with wild-type *S. aureus* (American Type Culture Collection number 25923), but all the isolates were more resistant than defensin-sensitive strains lacking cell-wall charge modifications that confer resistance.²⁰ The relative resistance of *S. aureus* isolates from patients with atopic dermatitis argues against the reversion of the bacteria to less resistant forms in this disorder. When these resistant strains of *S. aureus* were exposed to antimicrobial peptides expressed in psoriatic skin, however, the presence of both peptides enhanced their cytotoxic activity (Fig. 6). A combination of HBD-2 (at $80 \mu\text{M}$ or $160 \mu\text{M}$) and LL-37 (at $4 \mu\text{M}$) killed significantly more *S. aureus* organisms than did HBD-2 alone.

DISCUSSION

Atopic dermatitis is a very common skin disease known to be associated with a high prevalence of skin infections, particularly with *S. aureus*.¹³ The density

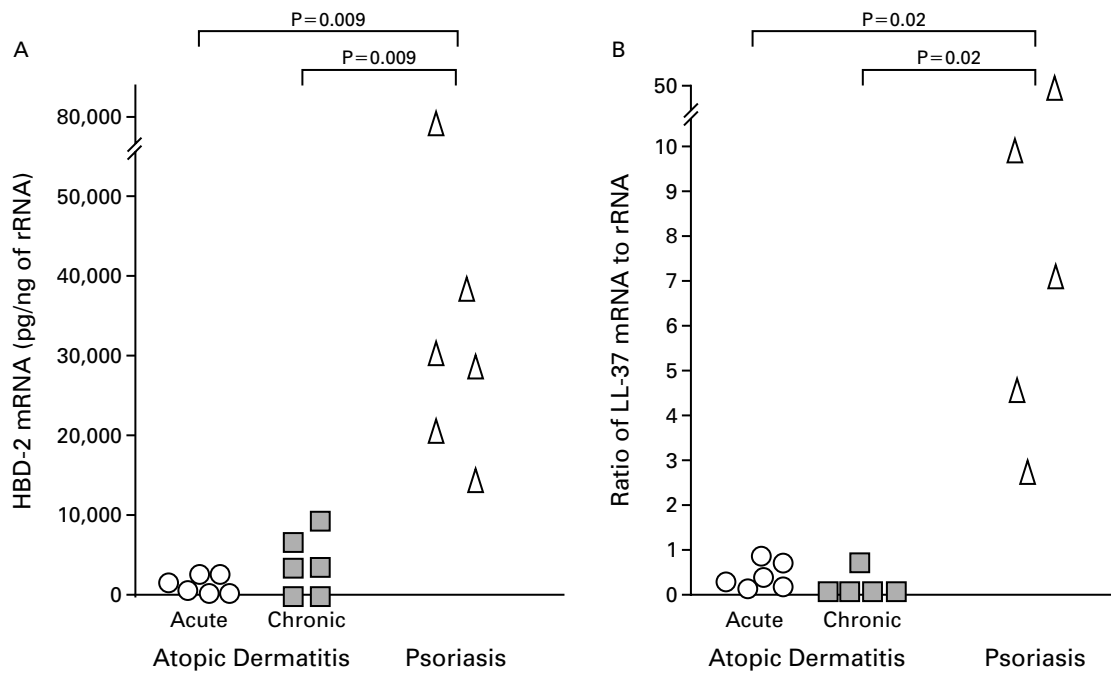


Figure 4. HBD-2 and LL-37 Messenger RNA (mRNA) in Atopic and Psoriatic Skin Lesions. Data are based on real-time reverse-transcriptase–polymerase-chain-reaction analyses of HBD-2 (Panel A) and LL-37 (Panel B). The abbreviation rRNA denotes ribosomal RNA.

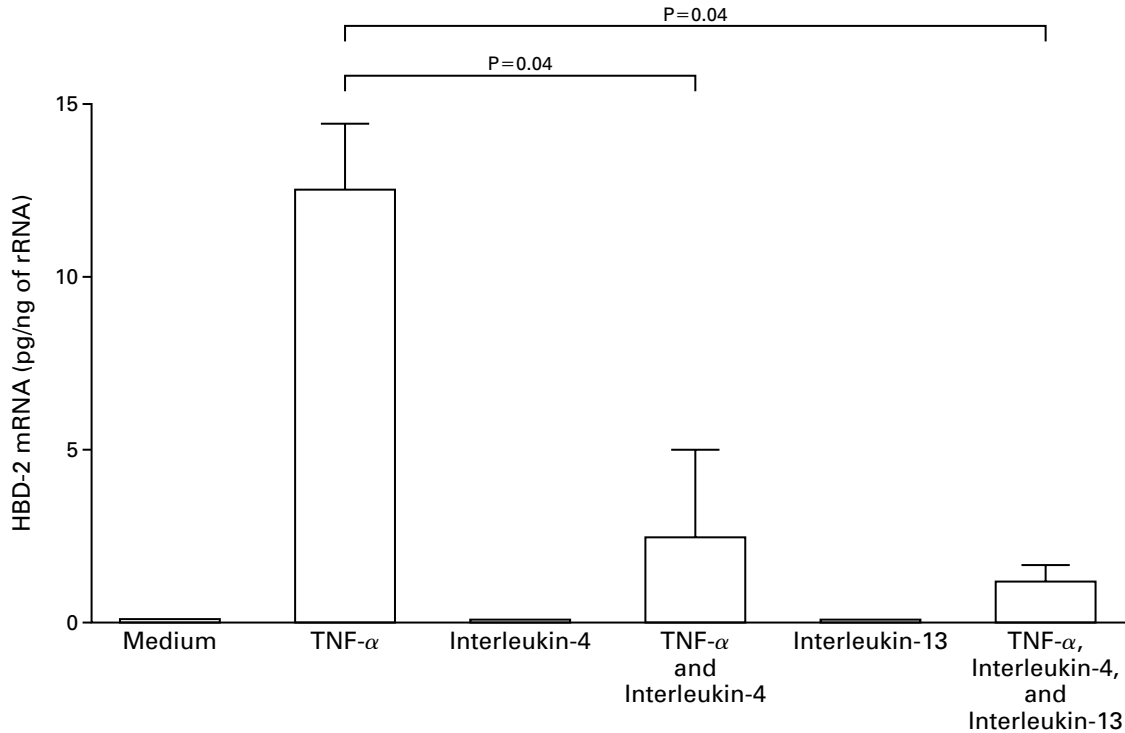


Figure 5. Effects of Interleukin-4 and Interleukin-13 on Tumor Necrosis Factor α (TNF- α)–Induced HBD-2 Expression in HaCat Cells. The concentration of TNF- α was 20 ng per milliliter, and the concentrations of interleukin-4 and interleukin-13 were 50 ng per milliliter each. The data are based on a total of three experiments. The bars indicate standard deviations, and rRNA denotes ribosomal RNA.

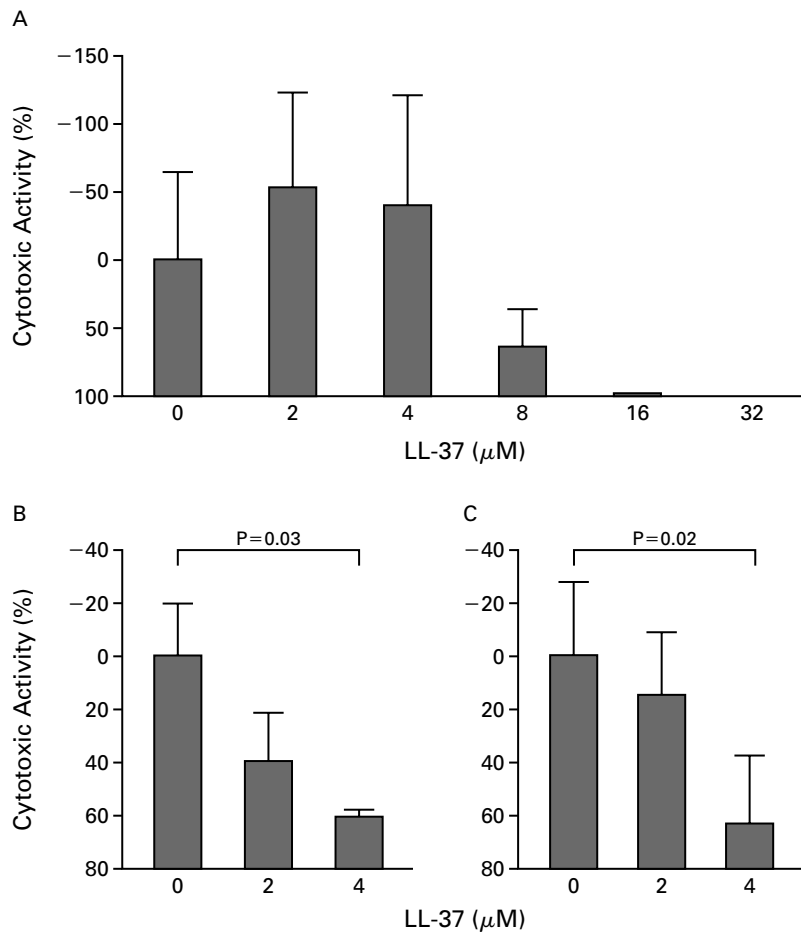


Figure 6. Cytotoxic Activity of HBD-2 and LL-37 against Wild-Type *Staphylococcus aureus*. Wild-type *S. aureus* (2.0×10^7 per milliliter) was incubated with increasing concentrations of LL-37 alone (Panel A) or LL-37 combined with HBD-2 at a concentration of $80 \mu\text{m}$ (Panel B) or $160 \mu\text{m}$ (Panel C). The I bars denote standard deviations.

of *S. aureus* in inflamed atopic lesions without clinical superinfection can be as high as 10^7 colony-forming units per square centimeter of lesional skin. The important role of *S. aureus* is supported by the observation that even in patients with atopic dermatitis who do not have superinfection, the severity of the skin disease is reduced by treatment with a combination of antistaphylococcal antibiotics and topical corticosteroids.²¹

Since *S. aureus* infection is a frequent trigger for the exacerbation of skin disease, there has been considerable interest in the mechanisms underlying the increased colonization of atopic skin lesions with *S. aureus*.²² Atopic skin has significantly greater binding affinity for *S. aureus* than nonatopic or psoriatic skin, probably as the result of underlying inflammation.²³ This view is supported by the observation that treat-

ment with topical glucocorticoids or tacrolimus reduces *S. aureus* counts on atopic skin.^{24,25} Indeed, a recent study has shown that the number of *S. aureus* organisms that bind to inflammatory skin lesions is significantly higher when the inflammation is mediated by type 2 helper T cells than when it is mediated by type 1 helper T cells.²⁶

The increased avidity of *S. aureus* for atopic skin, however, can account only for a several-fold increase in *S. aureus* on atopic skin.²³ Examination of skin-biopsy samples from patients with atopic dermatitis has shown that *S. aureus* grows in colonies in the upper layers of the epidermis between keratinocytes.²⁷ This suggests that an exponential increase in *S. aureus* could result from failure of the innate immune defense system of atopic skin to restrict the growth of the organisms. Naturally occurring antimicrobial peptides

are a critical component of this innate immune system that have been shown to provide mammalian skin with resistance to bacterial infection.¹¹ The antimicrobial peptides HBD-2 and LL-37 are normally produced by keratinocytes in response to inflammatory stimuli such as psoriasis or injury.⁷ Consistent with the concept that the activity of keratinocytes in atopic dermatitis differs from that in psoriasis, recent observations indicate that in atopic dermatitis, but not psoriasis, keratinocytes produce a distinct profile of chemokines that promote the influx of eosinophils and type 2 helper T cells into the skin, whereas psoriatic keratinocytes promote infiltration by neutrophils and type 1 helper T cells.²⁸

In our study, several independent approaches demonstrated that at both the protein level and the mRNA level, LL-37 and HBD-2 are deficient in skin lesions from patients with atopic dermatitis. We also demonstrated that the combination of HBD-2 and LL-37 at the concentrations found in psoriatic lesions, but not atopic lesions, was sufficient to kill *S. aureus*. These antimicrobial peptides have activity not only against bacteria but also against fungi and viruses. Thus, our observations may explain the increased susceptibility of patients with atopic dermatitis to fungal and viral infections such as herpes simplex and molluscum contagiosum.

We also investigated the potential mechanism (or mechanisms) for the reduced expression of antimicrobial peptides such as HBD-2. Since it is well established that atopic skin lesions are associated with increased expression of interleukin-4 and interleukin-13, we explored the possibility that these type 2 helper cytokines may inhibit HBD-2 gene expression. Indeed, we found that the combination of interleukin-4 and interleukin-13 was highly effective in inhibiting HBD-2 gene expression. Thus, type 2 helper cytokines account not only for the high IgE concentrations and eosinophilia in patients with atopic dermatitis but also for their increased susceptibility to skin infections.

In summary, our study showed that inflammatory skin lesions from patients with atopic dermatitis had significantly lower concentrations of HBD-2 and LL-37 than skin lesions from patients with psoriasis. The inhibitory effects of interleukin-4 and interleukin-13, both of which are abundant in atopic skin, may account for this finding. Furthermore, the low concentrations of HBD-2 and LL-37 in the patients with atopic dermatitis were unable to kill *S. aureus*. Therefore, a deficiency in the expression of inflammation-induced antimicrobial peptides may explain the susceptibility of patients with atopic dermatitis to skin infections. Our findings demonstrate the presence of skin-localized immunodeficiency in atopic dermatitis and the correlation between deficient expression of antimicrobial peptides and infection. These findings

point to the role of antimicrobial-peptide function in clinical disease and highlight the importance of considering the interaction between the innate and adaptive immune systems.

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