The epithelia constitute a major barrier to the environment and provide the first line of defense against invading microbes. Antimicrobial peptides are emerging as participants in the defense system of epithelial barriers in general. Originally we isolated the human antimicrobial peptide LL-37 from granulocytes. The gene (CAMP or cathelicidin antimicrobial peptide) coding for this peptide belongs to the cathelicidin family, whose members contain a conserved pro-part of the cathelin type. The human genome seems to have only one gene of this family, whereas some mammalian species have several cathelicidin genes. In the present work we demonstrate up-regulation of this human cathelicidin gene in inflammatory skin disorders, whereas in normal skin no induction was found. By in situ hybridization and immunohistochemistry the transcript and the peptide were located in keratinocytes throughout the epidermis of the inflammatory regions. In addition, the peptide was detected in partially pure fractions derived from psoriatic scales by immunoblotting. These fractions also exhibited antibacterial activity. We propose a protective role for LL-37, when the integrity of the skin barrier is damaged, participating in the first line of defense, and preventing local infection and systemic invasion of microbes.

Epithelia provide a barrier between the body and the environment. In addition, the epithelial cells have an active immunological role with antigen processing and presentation and production of cytokines and defense effector molecules such as microbicidal peptides. Thus, the epithelia mediate an active protection against invading microbes (1).

Several broad spectrum microbicidal peptides have been identified in mammalian mucosal epithelium; bovine tracheal mucosa produces a β-defensin, TAP (tracheal antimicrobial peptide) (2), paneth cells of the gastrointestinal mucosa of the skin and in inflammatory dermatoses. The RT-PCR1 experiment showed that the gene is not expressed in normal skin but interestingly in lesional psoriasis and challenged nickel allergy, indicating an induction of LL-37 in these skin disorders. We have confirmed the induction on cellular level by in situ hybridization and immunohistochemistry in various distinct inflammatory skin diseases such as psoriasis, subacute lupus erythematosus, dermatitis herpetiformis, atopic dermatitis, and nickel contact hypersensitivity. In these diseases the expression of the gene for LL-37 is induced in keratinocytes within the inflammatory regions, whereas in normal skin the peptide is not found in epidermis. In addition, we have detected LL-37 in partially purified fractions from psoriatic scales.

The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders.

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β-defensin, LAP (lingual antimicrobial peptide) is expressed by bovine tongue epithelial cells (5). Thus, peptide antibiotics appear at the surface epithelium where they are likely to act as key components in the first line of defense and in the wound healing process (5). So far, all mammalian antimicrobial peptides identified at the mucosal interface belong to the defensin family. Defensins are cysteine-rich peptides folded in β-pleated sheets with a broad activity against bacteria, enveloped viruses, fungi, and parasites (6).

We have isolated a clone for a novel human antibacterial peptide and named the putative peptide FALL-39 (7). Recently the mature active peptide LL-37 (two amino acids shorter at the N terminus than the putative peptide) was isolated from granulocytes and characterized (amino acid sequence is shown in Fig. 2B) (8). The preproprotein of LL-37 has also been named human CAP18 by another group (9). In contrast to the defensins, LL-37 is a cysteine-free peptide that can adopt an amphipathic α-helical conformation. The preproprotein belongs to the cathelicidin protein family. The common nominator of this protein family is a well conserved prorregion of cathelin type, whereas the C-terminal domain is represented by highly variant antibacterial peptides (10). A pronounced variation is also noticed in different numbers of cathelicidin genes between mammalian species. So far, prepro-LL-37 is the only human cathelicidin characterized. The gene is expressed in bone marrow and testis, and the peptide and its proform have been localized in granulocytes (8, 11). In addition, we have detected LL-37 together with several other antibacterial peptides in human wound and blister fluids (12).

To investigate antibacterial activity at human epithelial surfaces we initiated studies on the expression of LL-37 in human skin and in inflammatory dermatoses. The RT-PCR1 experiment showed that the gene is not expressed in normal skin but interestingly in lesional psoriasis and challenged nickel allergy, indicating an induction of LL-37 in these skin disorders. We have confirmed the induction on cellular level by in situ hybridization and immunohistochemistry in various distinct inflammatory skin diseases such as psoriasis, subacute lupus erythematosus, dermatitis herpetiformis, atopic dermatitis, and nickel contact hypersensitivity. In these diseases the expression of the gene for LL-37 is induced in keratinocytes within the inflammatory regions, whereas in normal skin the peptide is not found in epidermis. In addition, we have detected LL-37 in partially purified fractions from psoriatic scales.

The abbreviations used are: RT, reverse transcription; PCR, polymerase chain reaction; bp, base pairs; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; HPLC, high performance liquid chromatography.
peptide is also seen in chromatographic fractions derived from normal skin, where LL-37 most likely originates from granulocytes. Our results show that LL-37 is induced during inflammation in human skin, which is consistent with a protective role for LL-37 as an effector molecule in the first line of defense.

EXPERIMENTAL PROCEDURES

Skin Tissue—Punch biopsies were obtained both from normal skin of healthy volunteers and untreated patients with different inflammatory skin conditions; psoriasis, nickel allergy (before and after challenge), subacute lupus erythematosus, dermatitis herpetiformis, and atopic dermatitis (Table I). For immunohistochemistry the biopsies were fixed in formalin and embedded in paraffin, but biopsies for in situ hybridization were frozen directly.

RNA Extractions, RT-PCR, and Characterization of the Products—Total RNA was extracted from skin biopsies using RNAzol B (Biotecx Laboratories, Inc.) according to the instructions from the manufacturer. The RNA was denatured at 90 °C for 5 min before the first strand cDNA synthesis and then chilled on ice. For the first strand synthesis random hexamer primers and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) were used in a reaction volume of 20 μl using recommended conditions. The reaction was incubated at 40 °C for 45 min, heated at 95 °C for 5 min, then diluted to 100 μl with water, and stored at −20 °C until used for PCR. The primers 5'-GAAGCCTGAG were used in the PCR reactions and were directed to the signal sequence and the 3'-untranslated region of the LL-37 mRNA, respectively. Included in the PCR reactions were primers directed to glyceraldehyde-3-phosphate dehydrogenase (G3PDH; CLONTECH) to ascertain the specificity of the immunostaining, immunoadsorption was performed in a solution of 500 μl with the anti-LL-37 (dilution 1:500) and 5 μl of LL-37 in different concentrations. The mixture was incubated at room temperature for 3 h prior to immunohistochemical analysis.

Immunohistochemistry—Formalin-fixed and paraffin-embedded biopsies were sectioned at 4 μm. To quench endogenous peroxidase activity, deparaffinized, rehydrated sections were treated with 0.3% H2O2 in methanol for 30 min at room temperature. After rinsing in phosphate-buffer saline the sections were digested with 0.1% trypsin (Sigma) at 37 °C for 30 min. All sections were stained according to the indirect peroxidase method (15) utilizing a Vectastain ABC kit elite (Vector Laboratories) and following the instructions of the manufacturer. Briefly, the sections were incubated with 1.5% normal goat serum for 20 min at room temperature and then incubated overnight at 4 °C with the polyclonal antibody (LL-37). Control sections from the same tissues were similarly processed and analyzed during the experiment except that no LL-37 antibody was added. To ascertain the specificity of the immunostaining, immunoadsorption was performed in a solution of 500 μl with the anti-LL-37 (dilution 1:500) and 5 μl of LL-37 in different concentrations. The mixture was incubated at room temperature for 3 h prior to immunohistochemical analysis.

Immunoadsorption—Detection of LL-37 immunoreactivity in the chromatographic fractions was performed with a dot-blot assay using a rabbit polyclonal antibody specific for LL-37 that was obtained by a standard immunization scheme using 100 μg of synthetic peptide mixed with Freund’s complete adjuvant (16). Western blot analysis—Fractions that showed immunoreactivity in the dot-blot assay were selected for separation on discontinuous SDS-polyacrylamide gel electrophoresis using 16.5% Tris-Tricine Ready Gel (Bio-Rad). The material in the gels was blotted onto nitrocellulose filters (Hybond-C, Amersham Corp.) by electrophoretic transfer as described previously (16). Immunoreactivity was detected with the LL-37-specific antibody (see above in immunoadsorption). The second antibody was an anti-rabbit IgG conjugated with alkaline phosphatase, obtained from Sigma. The filter was stained for enzyme activity in 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, and 5 mM MgCl2 containing 4-nitro blue tetrazolium chloride (0.2 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml), both purchased from Boehringer Mannheim.

Antibacterial Assay—Thin plates (1 mm thick) were poured with LB (Luria Bertani) broth supplemented with medium E (17), 1% agarose,
was detected in the epidermis of healthy skin (not shown).

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104 cells/ml of either one of the two test bacteria strains, Escherichia coli D21 and Bacillus megaterium Bm11. Small wells (diameter, 3 mm) were punched in the plates, and 3-mL cultures of the two test bacteria strains, Escherichia coli D21 and Bacillus megaterium Bm11, were added to each well. After overnight incubation at 30 °C, samples were applied in the wells. After overnight incubation at 30 °C, the diameters of inhibition zones were recorded with a ruler.

RESULTS

Detection of the LL-37 Transcript in Psoriasis and Nickel Contact Allergy by RT-PCR—From three individuals, five biopsies were taken and RNA was prepared. Two biopsies were taken from a psoriatic patient, one was taken from a psoriatic lesion, and another was taken from an uninvolved region. From a patient with nickel contact allergy, one biopsy was obtained from a healthy skin and one was taken from a provoked nickel hypersensitivity reaction. In addition, one biopsy was obtained from normal skin of a healthy volunteer. The RNA from each biopsy was used separately for cDNA synthesis. The cDNA was utilized as templates in the PCR reactions. Two specific primer pairs were used for detection of transcripts. One pair was directed to the LL-37 cDNA, and the other pair was directed toward G3PDH cDNA; the latter served as control for efficient cDNA synthesis. The PCR reactions were size fractionated on an 1.2% agarose gel, and bands of expected size (983 bp) were detected in all lanes for the control G3PDH, whereas a band of 547 bp corresponding to LL-37 cDNA was detected only in the sample from the psoriatic lesion and challenged nickel allergy, and for the G3PDH probe there are positive signals in all lanes.

and approximately 6 x 10⁴ cells/ml of either one of the two test bacteria strains. The identity of the bands was confirmed by high stringency Southern blot hybridization with specific probes for LL-37 and G3PDH cDNA, respectively (Fig. 1). These results show an induction of LL-37 in psoriasis and nickel allergy. The prominent bands for the control G3PDH may reflect the hyperproliferative nature of keratinocytes in these conditions.

Cellular Localization in situ Hybridization and Immunohistochemistry—To determine cellular localization for the induction of LL-37 observed by RT-PCR, we performed in situ hybridization on samples on lesional psoriasis and challenged nickel allergy. The cRNA probes used correspond to the main LL-37 coding part and the 3′-untranslated region (Fig. 2). In both conditions (psoriasis and nickel allergy) there was abundant signal for LL-37 mRNA in keratinocytes throughout the epidermis (Fig. 3, A and E), whereas no hybridization signal was detected in the epidermis of healthy skin (not shown). Control slides hybridized with the sense cRNA probe were

negative (Fig. 3B for psoriasis).

In accordance with data obtained by in situ hybridizations, immunohistochemical analyses demonstrated strong staining for LL-37 peptide in epidermal keratinocytes in psoriasis and nickel allergy reaction (Fig. 3, C and F). Furthermore, a similar pattern of LL-37 immunoreactivity in keratinocytes was found in various dermatoses such as subacute lupus erythematosus (Fig. 3D), dermatitis herpetiformis (not shown), and atopic dermatitis (Fig. 3F). Thus, by extending our studies comprising distinct inflammatory disorders, our results clearly show that LL-37 is consistently induced in skin epithelium in association with inflammation irrespective of the underlying pathogenic mechanism. In addition, confirming previous results (8) and serving as an internal positive control, there was abundant immunoreactivity for LL-37 in granulocytes in all samples of both healthy and affected skin (Fig. 3J, affected skin). When LL-37 was used to absorb the antibody, immunostaining decreased in a dose-dependent manner as the concentration of the peptide was increased, and at 20 μg/ml peptide concentration the staining was completely abolished (Fig. 3H).

Chromatography and Detection of LL-37 in Psoriatic Scales and Normal Skin—Psoriatic scales (0.85 g) and normal skin (12 g) were extracted and purified on Sep-Pak as described under “Experimental Procedures.” Lyophilized material eluted from Sep-Pak, 5.5 mg derived from the psoriatic scales, and 12.8 mg from the normal skin were dissolved in 0.5 and 1.2 ml of 0.1% trifluoroacetic acid, respectively. Chromatography of each sample was performed on an HPLC reversed-phase column equilibrated in 0.1% aqueous trifluoroacetic acid, and the elution was with a five-step gradient of acetonitrile in 0.1% trifluoroacetic acid: 1) from 0 to 15% over 5 min, 2) from 15 to 40% over 25 min, 3) isocratic at 40% for 10 min, 4) from 40 to 65% over 25 min, and 5) from 65 to 80% over 10 min. Fig. 4 shows the chromatographic profiles of the material derived from both psoriatic scales (Fig. 4A) and normal skin (Fig. 4B). The collected fractions were lyophilized and redissolved in 100 μl of water for psoriatic scales and 250 μl for normal skin. To monitor the presence of LL-37 immunoreactivity, 2 μl of the fractions were used for dot-blot immunoassay. Several positive fractions were detected both in the psoriasis material and in the normal skin preparation. The fractions with the most pronounced immunoreactivity were further analyzed by Western blot analysis. The results are shown in Fig. 5 for psoriatic scales and normal skin fractions number 33, 34, and 35, respectively. In fraction 35 from the psoriatic scales a clear band corresponding to LL-37 was detected, whereas faint bands for LL-37 were seen in the other fractions. Furthermore, immuno-
reactivity was also present in the high molecular weight regions. This immunoreactivity could originate from the unprocessed proform, binding, or aggregation of the peptide with larger proteins. Another possible explanation is that the antibodies cross-react with some other proteins.

**Antibacterial Activity**—Fractions 33–35 analyzed by Western blot were also tested for antibacterial activity. From each fraction equal amounts of protein material (20 μg) was loaded.

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**Fig. 3.** The expression of LL-37 is induced in inflammatory skin disorders. A, frozen sections of psoriasis were hybridized with an antisense cRNA probe specific for LL-37 mRNA and counterstained with methylene green. Intense signal for LL-37 mRNA is detected throughout the epidermis (e). B, no signal is seen in the serial section hybridized with the sense cRNA probe for LL-37. C, immunohistochemistry using polyclonal antibody against LL-37 on a formalin fixed section of psoriasis reveals strong immunoreactivity for LL-37 in the epidermis (e). D, sample of subacute lupus erythematosus shows distinct immunoreactivity for LL-37 in epidermal keratinocytes. E, frozen section of cutaneous nickel allergy reaction hybridized for LL-37 mRNA demonstrates signals for LL-37 in the epidermis. F, abundant immunoreactivity for LL-37 is seen in the epidermis of formalin fixed sections of challenged nickel allergy. G, there was no immunoreactivity for LL-37 in the epidermis of healthy skin. H, absorption with 20 μg/ml LL-37 peptide on a sample of subacute lupus erythematosus abolished the immunoreactivity (serial section with D). I, sample of atopic dermatitis with positive immunostaining for LL-37 in the epidermis (e). J, strong immunostaining is also seen in granulocytes in the same section as D. Scale bars: A, B, E–H, and I, 25 μm; C, D, and J, 10 μm.
into the wells of the assay plate and for comparison known amounts of synthetic LL-37 were included. Fig. 6 shows that all these fractions have antibacterial activity. Elevated activity was detected in the material derived from the psoriatic scales as compared with normal skin. Notably, the highest antibacterial activity was recorded in fraction 35 of the psoriatic scales, which corresponds to the strongest band for LL-37 in the Western blot. However, the strength of activity is comparable with mg of LL-37, whereas the band intensity on Western is weaker, indicating that this fraction contains additional components, enhancing the antibacterial activity.

Taken together, our results demonstrate the induction of the gene coding for LL-37 on both mRNA and protein levels in human epidermis during inflammation.

**DISCUSSION**

Several microbicidal peptides are expressed by surface epithelial cells in mammals. The emerging concept is that these peptides contribute to the protective barrier of the epithelia by killing invading microorganisms (5, 18). Human skin is the major epithelial barrier between the body and the environment and functions as an active immune organ (19). Antimicrobial peptides are rapidly activated and have a broad spectrum activity against microbes (20) and may contribute to the first line of defense in skin epithelia. To find out if the recently discovered human antibacterial peptide LL-37 is a part of the skin immune system, we analyzed the expression of the corresponding gene in normal skin and in inflammatory skin diseases. The present work demonstrates for the first time induction of an antimicrobial peptide in human skin during inflammation. Our results are consistent with a protective role for LL-37 against microbial invasion through a disrupted skin barrier working together with migratory inflammatory cells.

We initiated our studies with RT-PCR that showed an induction of LL-37 in two inflammatory skin disorders, psoriasis, and challenged nickel allergy but no expression in healthy skin (Fig. 1). By a series of in situ hybridizations and immunohistochemistry, the up-regulation of the gene coding for LL-37 was confirmed and localized to keratinocytes. The tissue sections
were from normal skin and different inflammatory dermatoses: psoriasis, nickel contact hypersensitivity, subacute lupus erythematosus, dermatitis herpetiformis, and atopic dermatitis. In all these inflammatory dermatoses a pronounced induction in keratinocytes for LL-37 is noted, whereas in healthy skin no signal was detected in the epidermis.

To further evaluate the induction and also screen for antibacterial activity, protein extracts were prepared from psoriatic scales and healthy skin. After separation of the extracts on HPLC, several chromatographic fractions contained antibacterial activity. In some of these fractions both from psoriatic scales and healthy skin, LL-37 immunoreactivity could be detected by a dot-blot assay. To track this immunoreactivity a Western blot analysis was performed and a clear band was detected in one psoriatic fraction, representing LL-37. In the other fractions shown in Fig. 5 only faint bands in the same region were found. The detection of faint bands on the Western blot of LL-37 in normal skin is not in agreement with the results obtained by the other detection methods (RT-PCR, in situ hybridization, and immunohistochemistry). Our conclusion is that the peptide detected in normal skin originates from residing granulocytes that harbor the peptide in their vacuoles. Another explanation for faint bands on Western blot is that trauma during the surgical procedure of the reduction plastic surgery may induce LL-37.

The fractions that contained LL-37 immunoreactivity were analyzed for antibacterial activity together with a dilution series of synthetic LL-37. Fraction 35 from the psoriatic scales, which contains the highest amount of LL-37 as determined by band strength on Western blot, also exhibited the highest antibacterial activity. Because 20 µg of dry weight of fraction 35 were used in the antibacterial assay as well as on Western blot, the zone diameter as compared with the reference of LL-37 is larger than expected if the activity is solely derived from LL-37. However, this fraction is not purified to homogeneity, and the antibacterial activity must be dependent also on additional components that enhance the bactericidal effect in this fraction.

Previously, we have localized the tissue-specific expression of the gene for LL-37 to testis and bone marrow (7). We have also characterized and sequenced the complete CAMP gene (8). In the promoter region we identified potential binding sites for the transcription factors, acute phase response factor and nuclear factor for interleukin-6 expression, indicating that these transcription factors are recruited when the gene is turned on. Interleukin-6 (IL-6) regulates the activation of these two transcription factors (21, 22), and accordingly we suggested that this cytokine is an important modulator of the CAMP gene expression. In fact, this cytokine plays a crucial role in local and systemic inflammation, acting as a major alarm inducer during infection and injury. IL-6 and its corresponding receptor are known to be synthesized by keratinocytes (23). In addition, IL-6 is expressed at high levels in lesional psoriatic skin in contrast to normal skin and has been proposed to affect the function of dermal inflammatory cells (24). One of the effects of IL-6 might be the up-regulation of the CAMP gene that we have documented here in five different inflammatory dermatoses. Consequently, this induction could enhance the antimicrobial defense armament of the disrupted barrier and thus provide an important shield against systemic invasion.

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