Cutaneous Injury Induces the Release of Cathelicidin Anti-Microbial Peptides Active Against Group A *Streptococcus*

Robert A. Dorschner,* Vasumati K. Pestonjamasp,* Seema Tamakuwala,† Takaaki Ohtake,* Jennifer Rudisill,* Victor Nizet,† Birgitta Agerberth,‡ Gudmunder H. Gudmundsson,§ and Richard L. Gallo*†

*Division of Dermatology, University of California San Diego, and VA San Diego Healthcare Center, San Diego, California, U.S.A.; †Department of Pediatrics, University of California San Diego, San Diego, California, U.S.A.; ‡Department of Medical Biochemistry and Biophysics, Karolinska Institute, §Microbiology and Tumorbiology Center, Stockholm, Sweden

Cathelicidins are a family of peptides thought to provide an innate defensive barrier against a variety of potential microbial pathogens. The human and mouse cathelicidins (LL-37 and CRAMP, respectively) are expressed at select epithelial interfaces where they have been proposed to kill a number of gram-negative and gram-positive bacteria. To determine if these peptides play a part in the protection of skin against wound infections, the anti-microbial activity of LL-37 and CRAMP was determined against the common wound pathogen group A *Streptococcus*, and their expression was examined after cutaneous injury. We observed a large increase in the expression of cathelicidins in human and murine skin after sterile incision, or in mouse following infection by group A *Streptococcus*. The appearance of cathelicidins in skin was due to both synthesis within epidermal keratinocytes and deposition from granulocyctes that migrate to the site of injury. Synthesis and deposition in the wound was accompanied by processing from the inactive prostorage form to the mature C-terminal peptide. Analysis of anti-microbial activity of this C-terminal peptide against group A *Streptococcus* revealed that both LL-37 and CRAMP potently inhibited bacterial growth. Action against group A *Streptococcus* occurred in conditions that typically abolish the activity of anti-microbial peptides against other organisms. Thus, cathelicidins are well suited to provide defense against infections due to group A *Streptococcus*, and represent an important element of cutaneous innate immunity.* ***Key words: bacteria/infection/keratinocytes/skin/wound healing. J Invest Dermatol 117:91–97, 2001***

The term "anti-microbial peptide" has been used to describe a large number of small proteins that can kill or inhibit the growth of a wide variety of microbes. Plants, insects, and animals have evolved to produce a great variety of such peptides that are thought to be protective against pathogenic microorganisms (Boman, 1998; Ganz and Lehrer, 1999). More recently, the principles of anti-microbial peptide defense observed in other species have been applied to understanding human resistance to disease. The diversity of anti-microbial peptides, however, many exhibiting alternative biologic functions or acting synergistically, has made the relative contribution of individual peptides to host defense difficult to determine.

Known mammalian anti-microbial peptides belong to four main groups: α-defensins, β-defensins, θ-defensins, and cathelicidins. Depending on the species, most of these peptides are found in leukocyte granules and at a variety of epithelia. Anti-microbial peptides are generally the product of post-translational processing from an immature preproprotein to an active mature peptide. Cathelicidins are the most structurally diverse anti-microbial gene family in this mature C-terminal domain (Zanetti *et al*, 1995), with mature products that include peptides rich in proline and arginine (i.e., PR-39), those that form disulfide bonds and β-sheet structures similar to defensins (i.e., protegrins), and α-helical peptides (i.e., LL-37). Unlike defensins, LL-37 is the only member of the cathelicidin gene family found in humans (Gudmundsson *et al*, 1996), and closely resembles CRAMP, the sole member of this gene family in mice (Gallo *et al*, 1997).

Anti-microbial peptides have been proposed to provide innate immune defense at the epithelial interface with the external environment. Consistent with this function, expression of β-defensins and cathelicidins occurs in cells directly exposed to microbial pathogens (Goldman *et al*, 1997; Stolzenberg *et al*, 1997; Bals *et al*, 1998). For example, mucosal epithelia of the airways express both β-defensins and cathelicidins, thus presenting a theoretical inhibitory environment for microbial growth. Cathelicidins and β-defensins each inhibit the growth *in vitro* of several different bacterial species that are potential respiratory pathogens. This anti-microbial activity, however, is highly sensitive in some cases to the culture conditions and easily inactivated by alterations in the ionic strength or the addition of other molecules present at the mucosal interface (Goldman *et al*, 1997; Bals *et al*, 1998; Johansson *et al*, 1998). Inactivation of anti-microbial peptides by high ionic strength environments has been proposed as a possible contributor to the pathogenesis of pulmonary infections in cystic fibrosis patients (Goldman *et al*, 1997); however, the sensitivity of anti-microbial peptides to inactivation casts some doubt on their efficacy as a primary defense system.

Manuscript received November 9, 2000; revised January 8, 2001; accepted for publication January 22, 2001.

Reprint requests to: Dr. Richard L. Gallo, Dermatology 111B, 3350 La Jolla Village Dr, San Diego, CA 92161, U.S.A. Email: Rgallo@VAPOP.UCSD.edu

0022-202X/01/$15.00 · Copyright © 2001 by The Society for Investigative Dermatology, Inc.

The function of cathelicidins in human skin is incompletely understood. Human epidermis does not synthesize significant amounts of anti-microbial peptides in a constitutive manner despite the high degree of potential microbial exposure. Production of β-defensins and cathelicidins has been seen in diseased skin (Frohm *et al*, 1997; Harder *et al*, 1997), but a relationship between expression and disease pathogenesis has not been established. Furthermore, many potential skin pathogens have not been studied for their susceptibility to anti-microbial peptides. In this study we investigated whether injury can initiate cathelicidin expression in the skin. We showed that after injury human and mouse skin rapidly increases expression of cathelicidins in the epidermis and within the wound itself. Analysis of the biologic activity of cathelicidin against the important human skin pathogen group A *Streptococcus* (GAS) demonstrated that cathelicidins are highly active against this organism. Unlike other potential pathogenic targets for anti-microbial peptides, the ability of cathelicidins to inhibit GAS growth is stable under a wide range of relevant environmental conditions. These observations suggest cathelicidins can play an important part in innate immune defense of wound repair.

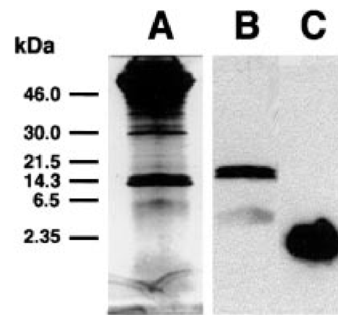

Figure 1. Antibody against CRAMP is specific. *Lane A*: silver-stained gel from murine bone marrow extract demonstrating multiple proteins present in extract. *Lanes B, C*: Western blots using an antibody against CRAMP described in *Materials and Methods*. *Lane B* contains bone marrow extract and *lane C* contains a synthetic CRAMP 16 amino acid fragment used as the immunogen. The antibody specifically recognized native peptides in the marrow extract corresponding to the predicted size of both the immature and mature cathelicidin peptide.

MATERIALS AND METHODS

Antibody production and western blot For antibody production a synthetic 16 amino acid CRAMP peptide fragment (GQKIKN-FIQKLVPQPE-OH) corresponding to amino acids 18–33 of the mature CRAMP protein was bound to Keyhole Limpet Hemocyanin and injected subcutaneously in rabbits (QCB, Hopkinton, MA). Rabbits were boosted four times with this peptide and bled 10 d after boosts. Anti-sera were checked by enzyme-linked immunosorbent assay and specific anti-CRAMP antibody purified via affinity chromatography over a sepharose column conjugated with CRAMP peptide fragment used as immunogen. To demonstrate specificity, affinity purified antibody was used to detect the 16 amino acid peptide fragment, or native CRAMP from murine bone marrow extracts. Bone marrow samples were boiled in sodium dodecyl sulfate (SDS) loading buffer containing 10% 2-mercaptoethanol for 5 min prior to electrophoresis. Proteins were separated via a 3.5–20% gradient SDS/polyacrylamide gel electrophoresis, then transferred on to a nitrocellulose membrane (MSI, Westborough, MA) or silver stained (Bio-Rad, Hercules, CA) for total protein. Rabbit antibodies were detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase (Dako A/S, Carpinteria, CA) and developed using chemiluminescence [(1 min incubation in 0.1 M Tris pH 8.5, 0.01% H_2O_2, 1.25 mM 3-aminophthalhydrazine (Fluka, Buchs, Switzerland), 0.25 mM coumaric acid (Sigma, St Louis, MO)]. Anti-CRAMP antibody was specific for two bands of ≈17 and 5 kDa, corresponding to the predicted sizes of the Pro-CRAMP and mature CRAMP, respectively (**Fig 1**, *lane B*). Similar methods using 4–20% Tris/Glycine gels (Novex, San Diego, CA) were used for western blots on normal or wounded skin extracts. Antibody to LL-37 was prepared as previously described (Gudmundsson *et al*, 1996).

Bacterial strains GAS strain NZ131 is a M49 serotype skin isolate from a patient who developed acute poststreptococcal glomerulonephritis (Simon and Ferretti, 1991). GAS strains 909 (M22), 317/98 (M59), and 1602/99 (M76) were kindly provided by Dr. Bernard Beall from recent epidemiologic investigations at the Centers for Disease Control in Atlanta. All GAS isolates possessed M protein gene chromosomal pattern "E" and produced the apolipoproteinase opacity factor as is characteristic for impetigo-causing strains (Bessen and Fischetti, 1990; Bessen *et al*, 1996). Group B streptococcal strains A909 (serotype Ia), M709 (Ib), DK23 (II), and K79 (III) were isolated from the blood or spinal fluid of septic neonates (Nizet *et al*, 1996, 1997). Group C streptococcal isolate VASD1 was isolated from the blood of an adult patient at the VA Medical Center, San Diego, CA.

Skin injury models Age- and sex-matched C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were anesthetized by inhalation of halothane, the hair of the back removed, and 1 cm full thickness incisions made through the panniculus carnosis. Mice were individually housed under aseptic conditions until analysis. For determination of cathelicidin expression, mice were killed via cervical dislocation at 6, 12, 24, 72, and 120 h postincision and a 1 cm area surrounding the wound excised and fixed in 10% buffered formalin. For extraction of total proteins used for western blotting of cathelicidins, crust from 120 h wounds, or unwounded skin, was similarly excised, pannicculus carnosis removed, tissue weighed, diced, and sonicated for 10 min in a volume of SDS loading buffer (4% SDS, 40% glycerol, 100 mM Tris pH 7.5, 0.05% Bromophenol Blue) proportional to its mass. In the GAS infection model, paraffin sections of murine skin were generously provided by Dr. Joyce C.S. De Azavedo at the University of Toronto. These hairless, 4 wk old male crl:SKH1(hrhr)Br mice were injected subcutaneously 24 h prior with 10^6 colony forming units of GAS NZ131(M49 serotype) complexed to Cytodex beads as a carrier, or with Cytodex beads alone (control), as previously described (Betschel *et al*, 1998). All procedures were approved by the VA San Diego Healthcare system subcommittee on animal studies.

Human skin wounds were collected following informed consent by inducing 0.5 cm full-thickness wounds in the axilla of healthy male volunteers under aseptic conditions with local anesthesia. At 24, 48, and 120 h after incision, 4 mm punch biopsies were taken from the site of injury and fixed in 10% buffered formalin. Apligraft cultured skin equivalents were generously provided by Dr. Tara Pouyani courtesy of Organogenisis (Canton, MA) and similarly fixed for histologic analysis.

Immunohistochemistry Paraffin-embedded formalin-fixed tissue sections were re-hydrated in a series of toluene, ethanol, and phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.5 mM KH_2PO_4, pH 7.3). Endogenous peroxidase activity was quenched with a 30 min incubation in 0.3% H_2O_2 in water, and sections microwaved for 5 min in antigen retrieval solution (0.01 M citric acid, 0.05 M NaOH pH 6.0). Sections were blocked with 2% goat serum in PBS then incubated with primary antibody (either 5 μg per ml rabbit anti LL-37 or 0.8 μg per ml anti CRAMP) in PBS, 0.1% bovine serum albumin. Sections were washed in PBS and detected with goat anti-rabbit-horseradish peroxidase (Vectastain ABC Elite Rabbit kit, Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate (Sigma) per manufacturers' instructions. In some cases sections were counterstained with hematoxylin after diaminobenzidine. Specificity of the primary antibody reaction was confirmed in separate experiments by adsorption of either anti-LL-37 or anti-CRAMP with excess amounts of the respective synthetic peptide. Specificity of the secondary antibody and immunostaining reagents was confirmed by routine use of rabbit preimmune sera.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) RNA was extracted as previously described (Chomczynski and Sacchi, 1987) from equal amounts of unwounded mouse skin, skin 2 or 12 h after a single 1 cm long sterile full-thickness incision, or from femural bone marrow. cDNA was made from 1 μg of each RNA with SuperScript II RNase H^- Reverse Transcriptase (Gibco BRL, Rockville, MD). PCR amplification of CRAMP was performed with oligonucleotides: CRAMP-F1 (GGATGAGAATAAATGAGGCTCTC) and CRAMP-B2 (TTTAGGAATCCAGAAACAGGC). Amplification of β-actin was performed with oligonucleotides BACTIN-1 (GGT-CGTCGACAACGGCTC) and BACTIN-2 (TGCCATGTTCAATG-GGGTAC). Reactions were run with 2 μl reverse transcription reaction template and 10 pmol oligos using Platinum PCR SuperMix (Gibco BRL, MD) in a 50 μl reaction volume. Thermal cycler profile: 94°C for

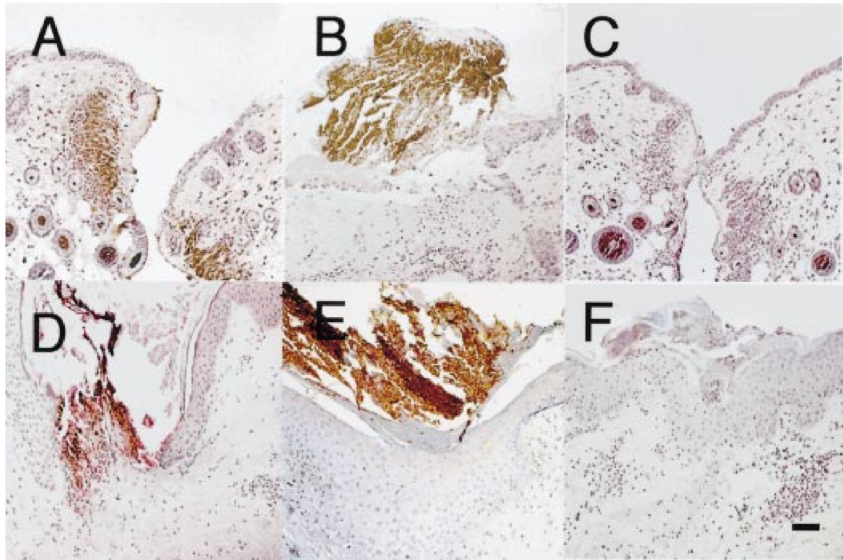

Figure 2. Cathelicidin is induced during wound repair. Expression of cathelicidins in murine and human skin wounds were evaluated with antibodies to CRAMP or LL-37. CRAMP expression was detected in murine wounds 6 h (*A*) and 5 d (*B*) after injury using antibody described in **Fig 1**. LL-37 was detected with anti-LL-37 in human wounds 24 h (*D*) and 5 d (*E*) after injury. Cathelicidins are seen in granulation tissue at the earliest time points examined after injury. By 5 d CRAMP (*B*) and LL-37 (*E*) are detected strongly in the crust, and faintly in the re-epithelialized epidermis. No staining was detected in murine or human sections with preimmune sera (C, murine; F, human). All sections were counterstained with hematoxylin as described in *Materials and Methods*. *Scale bar*: 40 μm.

5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; final extension step of 72°C for 7 min. This protocol was optimized for maximum visualization of product. Separate amplifications were performed for various cycles (20–35) to confirm consistency in relative detection between samples of CRAMP PCR products. Products were run on a 2.5% NuSieve 3:1 agarose gel (Biowhittaker Molecular Applications, Walkersville, MD) with Roche DNA molecular weight marker IX (X174 DNA cleaved with *Hae*III) and stained with ethidium bromide. For confirmation, the 607 bp PCR product was directly sequenced and found identical to CRAMP.

Peptide synthesis LL-37 peptide synthesis was performed with an Applied Biosystems model 430A peptide synthesizer using standard solid-phase procedures. Starting from t-butoxycarbonyl-Ser(benzyl)-OCH-phenylacetamidomethyl resin (0.67 mmol per g), t-butoxycarbonyl amino acid derivatives were used with reactive side chains protected as follows: serine and threonine, benzyl; lysine, 2-chlorobenzyloxycarbonyl; glutamate and aspartate, benzyl ester; and arginine, 4-toluenesulfonyl. Double couplings were performed for arginine, glutamine, and asparagine residues. The peptides were cleaved from the resins with liquid hydrogenfluoride/anisole/methylsulfide (10:1:1, vol/vol/vol) for 60 min at 0°C. The cleavage products were washed with diethylether to remove scavengers and protecting groups, extracted in 30% acetic acid, and lyophilized. The peptides were further purified by high-performance liquid chromatography (Waters, Milford, MA) on a reversed phase Vydac C18 column (The Separation Group, Hesperia, CA), using a linear gradient of acetonitrile (15–60% in 40 min) in 0.1% trifluoroacetic acid. The molecular masses were determined with a matrix-assisted laser desorption/ionization instrument (Lasermat 2000, Finnigan, San Jose, CA) and were, in all cases, in agreement with the calculated masses. Purification and N-terminal sequencing of the native mature mouse cathelicidin CRAMP from granulocytes has shown that this peptide is 33 amino acids with a N-terminus of GLL. Synthetic CRAMP peptide corresponding to the 33 amino acids: (GLLRKGGEKIGEKLKKIG-QKIKNFFQKLVPQPE) was synthesized commercially to > 95% purity as confirmed by high-performance liquid chromatography and mass spectrometry (Sigma Genosys, The Woodlands, TX).

Anti-microbial activity assays For assay of anti-microbial activity in solution, bacteria were grown in sterile tryptic soy broth (Sigma) to early log phase (OD_{600} = 0.06). In triplicate, 0.05 ml of bacterial suspension was added to wells of a 96 well flat bottomed tissue culture plate. Minimal inhibitory concentrations (MIC) of LL-37 or CRAMP were determined by 2-fold serial dilutions of each peptide. Bacterial growth was measured by recording the absorbance at 600 nm before and after an overnight incubation at 37°C. Percent inhibition of bacterial growth, as compared with controls containing no peptide, was calculated and MIC values determined based on ability to inhibit 80% or more of bacterial proliferation. Anti-microbial activity against *Escherichia coli* was also assayed in half-strength Mueller-Hinton broth containing Medium E (16 mM Na^+, 30 mM K^+, 0.8 mM Mg^{2+}) to compare anti-microbial activity of synthetic peptides to previously reported values for *E. coli* (Bals *et al*, 1998), and in Luria Broth.

RESULTS

Cathelicidins are induced in the skin following injury Cathelicidins are expressed in several tissues, including pulmonary epithelia, gut epithelia, testes, and bone marrow (Agerberth *et al*, 1991; Shi *et al*, 1994; Gallo *et al*, 1997; Sorensen *et al*, 1997; Bals *et al*, 1998; Wang *et al*, 1999), but have not been seen in the epidermis or dermis of normal skin (Frohm *et al*, 1997). To determine if cathelicidins are expressed in the skin following injury and, are therefore, available to provide innate anti-microbial defense following barrier compromise, sections of murine and human skin were first evaluated by immunohistochemistry at various time points after injury with antibodies specific to CRAMP or LL-37. Observation of both mouse and human wounds under low magnification showed abundant and similar patterns of expression (**Fig 2**). CRAMP, the sole known murine cathelicidin, was not detected in normal skin, but rapidly appeared following injury (**Fig 2*A*, *B***). Initially, CRAMP was detected most abundantly in the dermis at the edge of the wound, and later in the granulation tissue and crust. Expression of CRAMP in the dermis was mostly located extracellularly with the exception of staining seen within granulocytes. After re-epithelialization was complete, CRAMP was no longer detected (data not shown). Human skin immunostained for the human cathelicidin LL-37 revealed a similar pattern of expression. LL-37 was observed initially only at the site of injury and later in the granulation tissue and crust (**Fig 2*D*, *E***). Specificity of the antibody staining was confirmed on control sections stained with preimmune sera (**Fig 2*C*, *F***). These sections showed no staining and were similar to results obtained previously with these antibodies following preadsorption with excess LL-37 or CRAMP. Thus, wounding results in the accumulation of cathelicidin in the skin of humans and mice.

Under high-power magnification, LL-37 was evident within the keratinocytes at the wound edge, as well as in granulocytes (**Fig 3*A*, *B***), suggesting both cell types may contribute to the production of cathelicidin after injury. To confirm that keratinocyte cathelicidin staining was the product of endogenous production and not due to uptake of material originally synthesized by granulocytes, cultured skin equivalents comprised only of keratinocytes and fibroblasts were also stained with anti-LL-37. These cultured skin equivalents showed abundant staining for LL-37 in the upper granular layer and stratum corneum in a pattern similar to that seen at the wound edge (**Fig 3*C***).

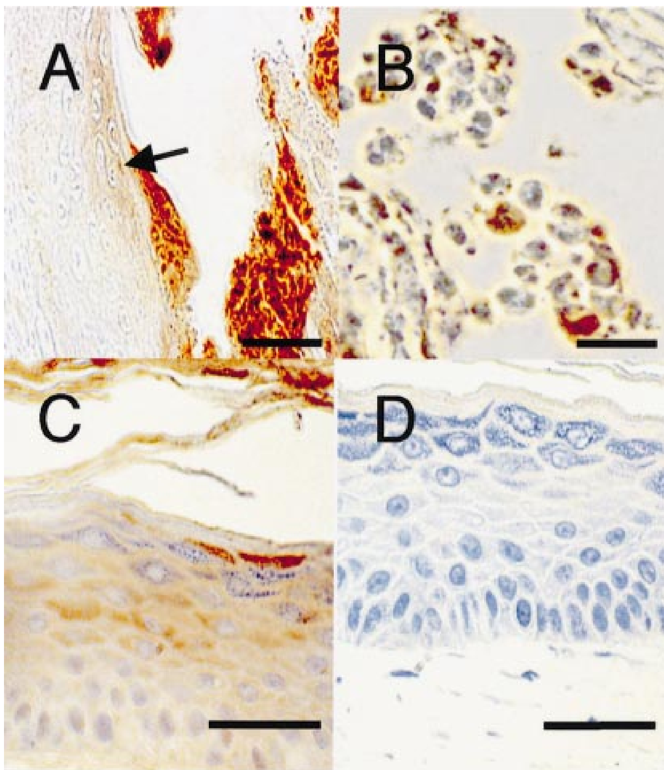

Figure 3. Cathelicidin is present in granulocytes and keratinocytes during wound repair. Human wounds 24 h after injury were examined at high power (× 630) to determine the location of cathelicidin expression. (*A*) LL-37 was detected in the keratinocytes (*arrow*) at the site of injury, and (*B*) abundantly in granulocytes. (*C*) Cultured human skin construct (Apligraf) stained for LL-37 as in **Fig 2** shows LL-37 expression in the granular layer of keratinocytes and stratum corneum after differentiation of the epidermis. Staining was not seen with preimmune sera (*D*). *Scale bar*: (*A*, *C*, *D*) 40 μm; (*B*) 20 μm.

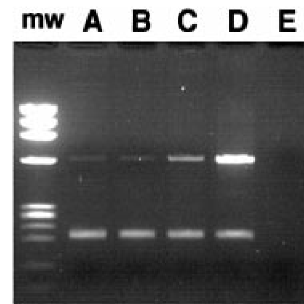

Figure 4. Cathelicidin mRNA is induced in wounds after injury. Total RNA was extracted from equal amounts of murine skin prior to injury (*A*), and 2 or 12 h after injury (*B*, *C*). Reverse transcription–PCR was performed on equal amounts of RNA extracted from each of these samples and from normal bone marrow as positive control (*D*). (*E*) Water as negative control. The upper band of 607 bp corresponds to the predicted size of CRAMP cDNA and was present more abundantly 12 h after injury or in RNA extracted from bone marrow used as a positive control. The lower band of 202 bp corresponds to the predicted size of the mouse β-actin PCR product that was simultaneously amplified in each reaction to demonstrate similar template availability. Molecular weight (Mw) marker X174 DNA cleaved with *Hae*III. CRAMP PCR products were sequenced to confirm identity.

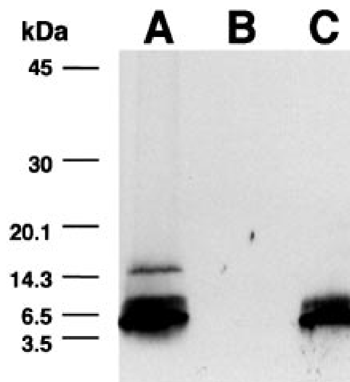

Figure 5. Cathelicidin is present in the mature form in wounds. Western blots were done as described in **Fig 1** with antibody against CRAMP on similar amounts (≈ 0.2 μg protein) of tissue extracts prepared from normal murine skin and skin 5 d after injury. *Lane A* contains the extract from wounded skin. *Lane B* contains extract from normal skin and *lane C* contains 10 pg pure synthetic CRAMP. A major band corresponding to the size of the mature peptide was seen in injured skin extracts. CRAMP was not detectable in the normal skin. Identical results were seen when 10 times more normal skin extract was loaded per lane.

Cathelicidin mRNA is induced after injury To evaluate whether cathelicidin induction was occurring at the transcriptional level, total RNA was prepared from normal skin, skin from the wound edge 2 or 12 h after injury, and bone marrow. Prior work with cathelicidin transcripts has shown they are present in many tissues, including at high levels in bone marrow, but are undetectable or at low levels in circulating blood or mature granulocytes (Agerberth *et al*, 1995; Gallo *et al*, 1997). CRAMP mRNA was detected at low levels in RNA from normal skin or skin 2 h after injury, but was abundantly present in skin 12 h after injury or in bone marrow that is known to contain abundant CRAMP mRNA (**Fig 4**). The presence of increased transcript in injured skin is similar to the increase in cathelicidin protein detected in keratinocytes by immunostaining.

Cathelicidin in wounds is processed to the mature form Cathelicidins are made as a propeptide with an anionic pro-domain known as the "cathelin" region N-terminal to the mature cationic anti-microbial peptide. Anti-microbial activity of cathelicidins present in granulocytes can be activated by elastase cleavage to free the C-terminal active peptide fragment (Zanetti *et al*, 1990; Shi and Ganz, 1998). Extraction of murine bone marrow prior to activation and analysis of CRAMP by SDS/polyacrylamide gel electrophoresis and western blot demonstrated that the majority of immunoreactive material is seen as the larger propeptide (**Fig 1**), a pattern typical for cathelicidins extracted from normal tissue (Wu *et al*, 1999). Additional experiments were performed to determine if CRAMP present in the wound was processed to the mature 33 amino acid peptide. Five days after wounding, murine wounds and normal skin controls were extracted and analyzed via western blot (**Fig 5**). Extracts from wounds showed abundant CRAMP as the mature form and little at the expected size of the propeptide (**Fig 5**, *lane A*). Consistent with results from immunohistology, blots of extracts from normal skin had no detectable CRAMP (**Fig 5**, *lane B*). Extracts of crust from human wounds similarly showed abundant LL-37 in the mature form (data not shown). Based upon comparison of the intensity of immunoreactive bands between crust extracts and a known amount of synthetic peptide, the approximate amount of CRAMP present in the wound is 0.1 μg per mg total protein.

CRAMP is induced in response to GAS infection GAS is an important human pathogen responsible for a variety of cutaneous infections, including impetigo, cellulitis, erysipelas, and wound infections. A murine model of GAS cutaneous infection was studied to determine if cathelicidin expression was also induced

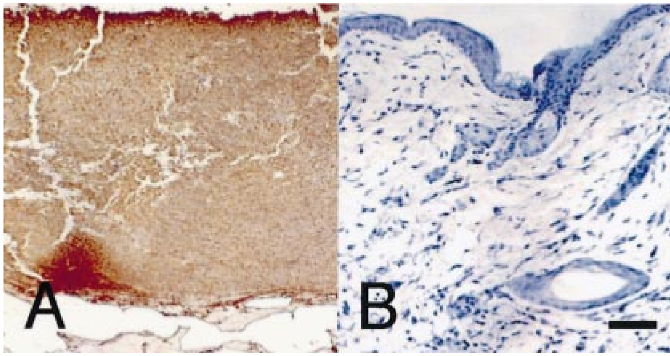

Figure 6. Group A Streptococcal infection induces cathelicidin expression. Mice were injected subcutaneously with 10^6 cfu of GAS strain NZ131 (M49) with Cytodex beads as carrier or with an equal number of Cytodex beads alone as control. One day after injection skin sections were immunostained as in **Fig 2** for CRAMP. (*A*) Abundant CRAMP was detected in the crust of skin injected with Cytodex beads coated with GAS. (*B*) CRAMP was not detected in skin following injection of Cytodex beads alone. *Scale bar*: 20 μm.

Table I. Anti-microbial activity of human and mouse cathelicidins[a]

Organism and strain	MIC (μM) LL-37	MIC (μM) CRAMP
Group A *Streptococcus*		
909 (serotype M22)	2	8
NZ131 (serotype M49)	2–16	8–16
317/98 (serotype M59)	1	4
1603/99 (serotype M76)	1	2
Group B *Streptococcus*		
A909 (serotype Ia)	>32	>32
M709 (serotype Ib)	>32	>32
DK23 (serotype II)	>32	>32
K79 (serotype III)	32	>32
Group C *Streptococcus* (strain SS-188)	16	32
Staphylococcus aureus ATCC no. 25923	>32	>32
Escherichia coli ATCC no. 25922	>32	>32

[a]Minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that inhibited at least 80% of growth after overnight incubation at 37°C in tryptic soy broth. Results were determined on triplicate samples and each experiment repeated three times.

following microbial invasion. Twenty-four hours after inoculation of GAS mixed with Cytodex beads, mice developed a localized ulcer with CRAMP abundantly expressed in the crust (**Fig 6**). This large deposition of cathelicidin persisted in the skin injured by GAS for up to 5 d. Cytodex beads alone injected as a control produced a minor foreign body reaction but no detectable increase in CRAMP in mice.

Cathelicidins are potent anti-microbial agents against GAS The induction of cathelicidins in skin following injury or GAS infection suggests its presence could influence the wound repair process. Previous work, however, has shown the anti-bacterial activity of many peptides is active only under optimized culture conditions, and only against bacteria that are relatively rare skin pathogens. At physiologic salt concentrations CRAMP, LL-37, and β-defensins are much less active against most bacteria and inactive against gram-positive organisms such as *Staphylococcus aureus* (Gallo *et al*, 1997; Bals *et al*, 1998; Johansson *et al*, 1998). For the expression of cathelicidins in skin to be beneficial as anti-microbials in the wound repair process, these peptides should inhibit the growth of relevant wound pathogens in this environment. To evaluate this, the anti-microbial action of LL-37 and CRAMP was determined against GAS in culture media (tryptic soy broth, Luria Broth) designed for optimization of bacterial growth (Chapin *et al*, 1999) as well as media previously used for anti-microbial peptide assays (0.5 × Mueller-Hinton + Medium E). Purity of each synthetic peptide was confirmed by high-performance liquid chromatography analysis. Peptides were also tested against known susceptible and resistant strains of *E. coli* and *S. aureus* and to confirm their bioactivity.

The human and murine cathelicidins potently inhibited the growth of GAS. Low concentrations of CRAMP and LL-37 inhibited growth of most GAS serotypes tested (**Table I**). Less activity was observed against groups B and C *Streptoccoci*. LL-37 and CRAMP were active against GAS in all culture media tested, not just those optimized for anti-microbial action. Bacteria such as *E. coli* that have been previously shown to be highly susceptible to LL-37 and CRAMP (MIC 0.15–1.5 μM), were not inhibited by LL-37 or CRAMP when assayed in these culture conditions. Thus, GAS is more sensitive to the action of these peptides under these culture conditions than bacteria previously reported to be highly sensitive. Additional evidence for the highly potent and stable action of cathelicidins against GAS was seen by further attempting to inactivate LL-37 by the addition of NaCl or serum. Inhibition of *E. coli* growth by LL-37 is abolished upon addition of serum or in increased salt conditions. Addition of up to 300 mM NaCl to culture media only slightly increased MIC values for LL-37 against GAS from 2 to 4 μM. One percent serum reduced the ability of 1 μM LL-37 to inhibit the of the growth of GAS from > 90% to 50%, but did not abolish activity. Thus, unlike bacterial strains previously evaluated, cathelicidins inhibit GAS growth under a range of culture environments.

DISCUSSION

The purpose of this study was to evaluate the expression and function of cathelicidins following injury to mammalian skin. Small cationic peptides such as the cathelicidins have been hypothesized to act as important components of innate immunity, providing an anti-microbial barrier against infection (Ganz *et al*, 1985; Zaslof, 1987; Lehrer *et al*, 1993; Bevins, 1994; Boman, 1996; Gallo and Huttner, 1998). Initial support for this hypothesis was based on *in vitro* observations that members of several anti-microbial gene families can kill a wide range of bacteria. Further support of the barrier hypothesis derives from the observation that epithelial surfaces, such as respiratory and gastrointestinal mucosa, express these peptides (Goldman *et al*, 1997; Agerberth *et al*, 1991, 1999; Jones and Bevins, 1992; Bals *et al*, 1998). Despite the potent *in vitro* anti-microbial activity and their appropriate tissue distribution, however, the argument for epithelial innate immunity by cathelicidins and defensins has been limited by relatively poor activity of many of these peptides when studied under culture conditions that more closely resemble those encountered *in vivo* (Bals *et al*, 1998; Johansson *et al*, 1998). Furthermore, the skin, the major epithelial organ exposed to the external environment, has minimal expression of cathelicidins under normal conditions (Frohm *et al*, 1997). In our study we show human LL-37, and its murine homolog CRAMP, are both rapidly expressed in the skin at the site of injury. Expression occurred in response to a sterile surgical incision or infection with GAS, and was characterized by increased mRNA together with increased mature bioactive cathelicidin. LL-37, or CRAMP, was produced by keratinocytes and present in granulocytes within the wound. These cathelicidins were further shown to have potent anti-microbial action against GAS, an important skin wound pathogen. This anti-microbial activity was not previously known for the cathelicidins.

Cathelicidins are a diverse group of anti-microbial peptides characterized by homology in the N-terminal pro-domain and great diversity in the C-terminal anti-microbial peptide region (Zanetti *et al*, 1995). Cathelicidins have been described in pig (Agerberth *et al*, 1991), rabbit (Ooi *et al*, 1990), cow (Selsted *et al*,

1992; Scocchi *et al*, 1997), sheep (Huttner *et al*, 1998), horse (Scocchi *et al*, 1999), mouse (Gallo *et al*, 1997), and humans (Agerberth *et al*, 1995). The structures of the mature C-terminal peptides vary between those rich in particular amino acids (PR-rich: BAC5, BAC7, PR-39; R-rich: P15s; W-rich: indolicidin), those forming β-sheets with disulfide bonds (protegrins) and those that are cysteine free and form α-helical structures [LL-37, CAP18 (Larrick *et al*, 1994) CRAMP]. The function and the spectrum of anti-microbial activity of cathelicidin family members differs, and some, like the PR-rich cathelicidins, act also to modify host cell functions (Gallo *et al*, 1994; Shi *et al*, 1996; Korthuis *et al*, 1999). Because of this structural and functional diversity, it is important to compare carefully the cathelicidins when extrapolating from animal models to humans. The murine cathelicidin CRAMP has proven to be a particularly useful model because it is similar in many respects to the human cathelicidin LL-37 (Gallo *et al*, 1997; Gudmundsson and Agerberth, 1999). Both represent the sole cathelicidin in their respective species, map to homologous chromosomal regions, have highly homologous 5′-untranslated regions, and have mature peptide domains that are structurally conserved with comparable spectra of anti-microbial activity (Gallo *et al*, 1997). Owing to their overall similarity, the expression pattern of both cathelicidins was evaluated in our study. In this fashion, observations made in human skin could be corroborated and further explored in a mouse model.

Nearly identical patterns of expression were observed for LL-37 and CRAMP. Immunoreactivity against cathelicidins in skin was seen only following injury and localized to the wound edge. Expression persisted for several days and formed a dense superficial boundary between the healing wound and the external environment. LL-37 and CRAMP were clearly seen in at least two cell types, neutrophils and keratinocytes. The expression of cathelicidins in granulocytes likely reflects prior synthesis of the stored propeptide for stimulus-induced release (Zanetti *et al*, 1990); however, new synthesis was observed for cathelicidins derived from keratinocytes. This was most apparent with LL-37, which appeared in keratinocytes at the wound edge. The production of cathelicidins in wounded skin resembled the expression seen in keratinocytes from cultured skin equivalents. Finding LL-37 expression in cultured skin equivalents confirms that detection of cathelicidins in whole skin is likely reflective of *de novo* synthesis and not just due to deposition from infiltrating granulocytes. These findings were also supported by the appearance of CRAMP transcripts in wounded mouse skin.

Keratinocytes are the likely source for these transcripts as mature granulocytes do not contain detectable levels of CRAMP mRNA (Gallo *et al*, 1997); however, multiple cell types may contribute to the production of cathelicidins following injury, both keratinocytes and granulocytes are significant sources for the anti-microbial peptide, and tissue-infiltrating granulocytes may increase CRAMP mRNA together with keratinocytes. As a whole tissue, injured skin expressed cathelicidin abundantly whereas uninjured skin did not. The potential for expression by keratinocytes enables rapid site-specific delivery independent of the time necessary for leukocyte recruitment. Interestingly, the ability of cultured skin equivalents to produce cathelicidins constitutively suggests that unlike normal skin, this tissue could act similar to wounded skin. The significance of cathelicidin expression in the wound and in skin equivalents lies in understanding its function.

To investigate the function of cathelicidins as a barrier to wound infection, the range of bacteria tested for susceptibility was increased to include additional wound pathogens. Prior studies of CRAMP and LL-37 have shown poor anti-microbial action (MIC > 64 μM) against the common skin pathogen *S. aureus*, better activity (MIC 4–16 μM) against the burn pathogen *Pseudomonas aeruginosa*, and best activity (MIC 5–16 μM) against *E. coli* and other species that rarely infect skin (Gallo *et al*, 1997; Bals *et al*, 1998; Johansson *et al*, 1998; Turner *et al*, 1998). In the most susceptible bacteria, LL-37 action was dependent on the optimization of assay conditions. Anti-microbial activity was inactivated when bound to serum elements or when assayed in standard bacterial culture media (Johansson *et al*, 1998; Wang *et al*, 1998). When assayed under the bacterial growth conditions used in this study, LL-37 and CRAMP both had poor activity against *E. coli* (**Table I**). These findings argue against action as a broad-spectrum anti-microbial agent despite observations of their anti-microbial activity in other assay systems (Travis *et al*, 2000), and finding that cathelicidins are present in wound fluids at concentrations sufficient to inhibit bacterial growth if assayed under optimal anti-microbial conditions (Frohm *et al*, 1996; Shi and Ganz, 1998).

Our results show that the cathelicidins are potent selective inhibitors of GAS, one of the most prevalent and potentially invasive skin pathogens. This activity was confirmed for four different GAS isolates belonging to characteristic serotypes associated with skin infection. The amount of cathelicidin present in the wound was estimated in the present study to be ≈ 0.1 μg per mg total protein. A conservative estimate of the total protein per volume available at the wound of 100 mg per ml yields a concentration of CRAMP as 3–10 μM, within the MIC. In contrast, LL-37 and CRAMP were not effective inhibitors of the closely related group B and C *Streptoccoci*, which more frequently occupy niches on the respiratory, gastrointestinal, or vaginal mucosa. The spectrum of GAS infections in skin range from self-limited impetigo to the potentially life-threatening necrotizing fasciitis. Relevant to its activity against this infectious skin organism, CRAMP is present in the skin after GAS infection and deposited within the scale crust of the necrotic lesion. This expression of cathelicidin could, therefore, reflect a specific defense mechanism by the skin to limit the proliferation of GAS. Alternatively, expression may be a nonspecific response to the tissue injury mediated by GAS. Given the induction of expression by sterile surgical incision, the latter explanation is more likely. Further work is required to explore the mechanisms responsible for regulation of cathelicidin expression.

Cathelicidins have been described to have several other functions in addition to anti-microbial activity such as induction of proteoglycan expression (Gallo *et al*, 1994) effects on neutrophil migration and chemotactic activity (Huang *et al*, 1997), and induction of apoptosis (Risso *et al*, 1998). These findings suggest the possibility that expression can function in wound repair through mechanisms that act directly on the cells of the wound environment and not solely against microbial pathogens. This hypothesis remains to be directly tested, but does not exclude the benefit to the wound repair process that deposition of a potent inhibitor of GAS growth offers.

In summary, this study demonstrates that cathelicidin expression is a characteristic of the response of the skin to injury. Functionally, the significance of this response lies (at least in part) in the newly recognized ability of human and murine cathelicidins to inhibit growth of GAS under conditions that would not permit anti-microbial function against other bacteria. Cathelicidins, possibly combined with other anti-microbial peptides, such as the newly described β-defensin-3 in the skin, suggest that evolutionarily ancient systems of innate defense by anti-microbial peptides can function in specialized situations during human wound repair. Understanding these events and their application may help in the diagnosis and therapy of several human diseases.

This work was supported by National Institutes of Health (NIH) grant AR-44379 and AI-48176 (RG) a grant from the American Skin Association (RG), a VA Merit Award (RG), The Wound Healing Society (VP), and the Swedish Medical Research Council (GG, BA)

REFERENCES

Agerberth B, Lee J, Bergman T, Cariquist M, Boman H, Mutt V, Jornvall H: Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur J Biochem* 202:849–854, 1991

Agerberth B, Gunne H, Odeberg J, Kogner P, Boman H, Gudmundsson G: FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci USA* 92:195–199, 1995

Agerberth B, Grunewald J, Castanos-Velez E, *et al*: Antibacterial components in bronchoalveolar lavage fluid from healthy individuals and sarcoidosis patients. *Am J Respir Crit Care Med* 160:283–290, 1999

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 anti-microbial activity at the airway surface. *Proc Natl Acad Sci USA* 95:9541–9546, 1998

Bessen D, Fischetti V: A human IgG receptor of group A streptococci is associated with tissue site of infection and streptococcal class. *J Infect Dis* 161:747–754, 1990

Bessen D, Sotir C, Readdy T, Hollingshead S: Genetic correlates of throat and skin isolates of group A streptococci. *J Infect Dis* 173:896–900, 1996

Betschel S, Borgia S, Barg N, Low D, De Azavedo J: Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. *Infect Immun* 66:1671–1679, 1998

Bevins C: Anti-microbial peptides as agents of mucosal immunity. *Ciba Found Symp* 186:250–260, 1994

Boman H: Peptide antibiotics: holy or heretic grails of innate immunity? *Scand J Immunol* 43:475–482, 1996

Boman HG: Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand J Immunol* 48:15–25, 1998

Chapin KC, Murray PR, *Media*. In Murray PR, Barom EJ, Phaller MA, Tenover FC, Yolken RH (eds). *Manual of Clinical Microbiology*, 7th edn. Washington, DC: ASM Press, 1999, pp 1687–1707

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, Gudmundsson GH: The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 272:15258–15263, 1997

Frohm M, Gunne H, Bergman A-C, *et al*: Biochemical and antibacterial analysis of human wound and blister fluid. *Eur J Biochem* 237:86–92, 1996

Gallo R, Huttner K: Anti-microbial peptides: an emerging concept in cutaneous biology. *J Invest Dermatol* 111:739–743, 1998

Gallo RL, Ono M, Povsic T, Page C, Eriksson E, Klagsbrun M, Bernfield M: Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich anti-microbial peptide from wounds. *Proc Natl Acad Sci USA* 91:11035–11039, 1994

Gallo RL, Kirn KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, Gennaro R: Identification of CRAMP, a cathelin-related anti-microbial peptide expressed in the embryonic and adult mouse. *J Biol Chem* 272:13088–13093, 1997

Ganz T, Lehrer R: Antibiotic peptides from higher eukaryotes: biology and applications. *Mol Med Today* 5:292–297, 1999

Ganz T, Selsted ME, Szklarek D, Harwig SSL, Daher K, Bainton DF, Lehrer RI: Defensins: natural peptide antibiotics of human neutrophils. *J Clin Invest* 76:1427–1435, 1985

Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson GM: Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88:553–560, 1997

Gudmundsson GH, Agerberth B, Odeberg J, Bergman T, Olsson B, Salcedo R: The human gene *Fall39* and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem* 238:325–332, 1996

Gudmundsson GH, Agerberth B: Neutrophil antibacterial peptides, multifunctional effector molecules in the mammalian immune system. *J Immunol Methods* 232:45–54, 1999

Harder J, Bartels J, Christophers E, Schroder J-M: A peptide antibiotic from human skin. *Nature* 387:861, 1997

Huang HJ, Ross CR, Blecha F: Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J Leukoc Biol* 61:624–629, 1997

Huttner K, Lambeth M, Burkin H, Burkin D, Broad T: Localization and genomic organization of sheep anti-microbial peptide genes. *Gene* 206:85–91, 1998

Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B: Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem* 273:3718–3724, 1998

Jones DE, Bevins CL: Paneth cells of the human small intestine express an anti-microbial peptide gene. *J Biol Chem* 267:23216–23225, 1992

Korthuis R, Gute D, Blecha F, Ross C: PR-39, a proline/arginine-rich anti-microbial peptide, prevents postischemic microvascular dysfunction. *Am J Physiol* 277:H1007–H1013, 1999

Larrick J, Hirata M, Zheng H, Zhong J, Bolin D, Cavaillon J, Warren H, Wright S: A novel granulocyte-derived peptide with lipopolysaccharide-neutralizing activity. *J Immunol* 152:231–240, 1994

Lehrer, Rl Lichtenstein AK, Ganz T: DEFENSINS: anti-microbial and cytotoxic peptides of mammalian cells. *Annu Rev Immunol* 11:105–128, 1993

Nizet V, Gibson RL, Chi EY, Framson PE, Hulse M, Rubens CE: Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. *Infect Immun* 64:3818–3826, 1996

Nizet V, Kim K, Stins M, Jonas M, Chi E, Nguyen D, Rubens C: Invasion of brain microvascular endothelial cells by group B streptococci. *Infect Immun* 65:5074–5081, 1997

Ooi CE, Weiss J, Levy O, Elsbach P: Isolation of two isoforms of a novel 15-kDa protein from rabbit polymorphonuclear leukocytes that modulate the antibacterial actions of other leukocyte proteins. *J Biol Chem* 265:15956–15962, 1990

Risso A, Zanetti M, Gennaro R: Cytotoxicity and apoptosis mediated by two peptides of innate immunity. *Cell Immunol* 189:107–115, 1998

Scocchi M, Wang S, Zanetti M: Structural organization of the bovine cathelicidin gene family and identification of a novel member. *FEBS Lett* 417:311–315, 1997

Scocchi M, Bontempo D, Boscolo S, Tomasinsig L, Giulotto E, Zanetti M: Novel cathelicidins in horse leukocytes. *FEBS Lett* 457:459–464, 1999

Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor JS: Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J Biol Chem* 267:4292–4295, 1992

Shi J, Ganz T: The role of protegrins and other elastase-activated polypeptides in the bactericidal properties of porcine inflammatory fluids. *Infect Immun* 66:3611–3617, 1998

Shi J, Ross C, Chengappa M, Blecha F: Identification of a proline-arginine-rich antibacterial peptide from neutrophils that is analogous to PR-39, an antibacterial peptide from the small intestine. *J Leukoc Biol* 56:807–811, 1994

Shi J, Ross C, Leto T, Blecha F: PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NADPH oxidase activity by binding to Src homology 3 domains of $p47^{phox}$. *Proc Natl Acad Sci USA* 93:6014–6018, 1996

Simon D, Ferretti J: Electrotransformation of *Streptococcus pyogenes* with plasmid and linear DNA. *FEMS Microbiol Lett* 66:219–224, 1991

Sorensen O, Amijots K, Cowland J, Bainton D, Borregaard N: The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 90:2796–2803, 1997

Stolzenberg ED, Anderson GM, Ackerman MR, Whitlock RH, Zasloff M: Epithelial antibiotic induced in states of disease. *Proc Natl Acad Sci USA* 94:8686–8690, 1997

Travis SM, Anderson NN, Forsyth WR, *et al*: Bactericidal activity of mammalian cathelicidin-derived peptides [In Process Citation]. *Infect Immun* 68:2748–2755, 2000

Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI: Activities of LL-37, a cathelin-associated anti-microbial peptide of human neutrophils. *Antimicrob Agents Chemother* 42:2206–2214, 1998

Wang Y, Agerberth B, Lothgren A, Almstedt A, Johansson J: Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. *J Biol Chem* 273:33115–33118, 1998

Wang Y, Griffiths WJ, Curstedt T, Johansson J: Porcine pulmonary surfactant preparations contain the antibacterial peptide prophenin and a C-terminal 18-residue fragment thereof. *FEBS Lett* 460:257–262, 1999

Wu H, Zhang G, Ross C, Blecha F: Cathelicidin gene expression in porcine tissues: roles in ontogeny and tissue specificity. *Infect Immun* 67:439–442, 1999

Zanetti M, Litteri L, Gennaro R, Horstmaim H, Romeo D: Bactenecins, defense polypeptides of bovine neutrophils, are generated from precursor molecules stored in the large granules. *J Cell Biol* 111:1363–1371, 1990

Zanetti M, Gennaro R, Romeo D: Cathelicidins: a novel protein family with a common proregion and a variable C-terminal anti-microbial domain. *FEBS Lett* 374:1–5, 1995

Zasloff M: Magainins, a class of anti-microbial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 84:5449–5453, 1987