Expression and Secretion of Cathelicidin Antimicrobial Peptides in Murine Mammary Glands and Human Milk

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ABSTRACT

Mammalian milk possesses inherent antimicrobial properties that have been attributed to several diverse molecules. Recently, antimicrobial peptides that belong to the cathelicidin gene family have been found to be important to the mammalian immune response. This antimicrobial is expressed in several tissues and increased in neonatal skin, possibly to compensate for an immature adaptive immune response. We hypothesized that the mammary gland could produce and secrete cathelicidin onto the epithelial surface and into milk. Human cathelicidin hCAP18/LL-37 mRNA was detected in human milk cells by PCR. Quantitative real-time PCR demonstrated an increase in relative expression levels at 30 and 60 d after parturition. Immunohistochemistry of mouse breast tissue identified the murine cathelicidin-related antimicrobial peptide in lobuloacinar and ductules. Western blot analysis of human milk showed that LL-37 was secreted and present in the mature peptide form. The antimicrobial activity of LL-37 against *Staphylococcus aureus*, group A *Streptococcus*, and enteroinvasive *Escherichia coli* O29 in the human milk ionic environment was confirmed by solution colony-forming assay using synthetic peptide. These results indicate that cathelicidin is secreted in mammary gland and human milk, has antimicrobial activity against both Gram-positive and Gram-negative bacteria, and can contribute to the anti-infectious properties of milk. (*Pediatr Res* **57:** 10–15, 2005)

Abbreviations
ACN, acetonitrile
AMS, autoclaved milk supernatant
CRAMP, cathelicidin-related antimicrobial peptide
GAS group A *Streptococcus* hBD, human β-defensin
hCAP18, human cationic antimicrobial peptide 18
RT-PCR, reverse transcriptase–PCR
TFA, trifluoroacetic acid

Antimicrobial peptides are found throughout nature and are important for the immune defense of plants, insects, and animals (1). Most of these molecules have a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses. In mammals, peptides from the cathelicidin family assist the antimicrobial efficacy of neutrophils, macrophages, and mast cells (2–6). In addition, antimicrobial genes of the defensin and cathelicidin families are produced by epithelia in response to injury of lung, gut, urinary bladder, oral mucosa, and skin (7–9). At the interface with the external environment, these molecules serve as a rapid first-line defense for inhibition of microbial proliferation and invasion.

Human milk contains various anti-infectious materials such as immunoglobulins, cellular components, and cytokines. It is believed that these molecules contribute to decreased morbidity and mortality of infants who are fed breast milk (10,11). Recently, peptide antibiotics have been isolated from human milk, providing additional evidence that multiple molecules can be involved in the immune defense of this material (12,13). In other systems, the clinical consequences of antimicrobial peptide expression have been observed in patients who have atopic dermatitis and are susceptible to infection and also lack the ability to increase the cathelicidin LL-37 and human β-defensin (hBD-2) in response to inflammatory stimuli (14). Furthermore, patients with Kostmann syndrome, a rare inherited disorder characterized by frequent infections and neutrophil dysfunction, also have a deficiency in production and processing of the human cathelicidin LL-37 (15). Such emerging clinical associations support the need to further explore the expression of antimicrobial peptides in specific aspects of the immune system.

The neonate produces increased levels of cathelicidin in the skin (16), suggesting that enhanced expression of a defense

peptide may compensate for an immature immune defense system. In this investigation, we evaluated expression of cathelicidins in human milk to determine whether the lactating mammary gland expresses this molecule, thus providing the neonate with this natural antibiotic by an oral route and protecting mammary epithelia from infection. Our data show that cathelicidin is transcribed by cells of the mammary gland and that the human peptide LL-37 is secreted in its active form.

METHODS

RNA and protein purification from human milk. The use of human subjects was approved by the UCSD institutional review board under protocol 021060XT. Volunteers were between 30 and 33 y of age, were in good health throughout pregnancy, and received no medication during pregnancy or lactation. These were our volunteers' first pregnancies, lasting 39–40 wk, with infants receiving complete breastfeeding. Excess discarded human milk was collected manually after feeding, captured in 50-mL conical polypropylene tubes (Corning, Corning, New York), and stored and transported at −20°C. Collections were at days 10, 15, 30, and 60 after parturition, and cellular elements were collected by centrifugation at $300 \times g$ for 15 min at 4°C. For protein purification, 20 mL of human milk was mixed with 20 mL of 1% trifluoroacetic acid (TFA) in 60% acetonitrile (ACN), incubated overnight at 4°C, then spun at $1000 \times g$ for 15 min at 4°C for collection of supernatant. Supernatant was filtered (0.20 μm, low binding; Fisher, Tustin, CA) and applied on to Sep-Pak C18 cartridges (Waters, Milford, MA) equilibrated with 0.1% TFA in doubly glass distilled water (DDW). Column was washed with 0.1% TFA, and bound material was eluted with 0.1% TFA in 80% ACN. Eluted fractions were concentrated by lyophilization and resuspended in 100 μL of doubly glass distilled water. All samples were stored at −20°C until use. Total protein concentration of the collected milk was measured by BCA assay (protein assay reagent; Pierce, Rockford, IL).

Reverse transcription–PCR. Total RNA was extracted from fresh cells and tissues using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was prepared from 1 μg of total RNA using RETROscript kit (Ambion, Woodward Austin, TX). PCR protocol for amplification of LL-37, hBD-1, and hBD-2 cDNA was as previously described (17). PCR amplification of LL-37 was performed with the forward primer 5'-GATAACAAGAGATTTGCCCTGCTG-3' and the reverse primer 5'-TTTCTCAGAGCCCAGAAGCCTG-3' for a 173-bp product. Amplification of 18S rRNA was done in parallel for all samples with QuantumRNA classic 18S internal standard kit (Ambion). The thermal cycler profile used was denaturation at 90°C for 10 min, followed by 35 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and extension at 72°C for 60 s.

Real-time quantitative PCR. Quantitative PCR was performed using a GeneAmp 7700 Sequence Detection System from Perkin Elmer. A 1-μL RT reaction was used in 23.5 μL of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 0.25 μL of each 10-μM primer described above. The thermal cycler profile used was 58°C for 2 min, 95°C for 10 min, and 40 cycles of amplification with denaturation at 95°C for 15 s and a combined annealing and extension step at 60°C for 1 min. Results were analyzed using the Comparative Ct Method, where Ct is the number of cycles required to reach an arbitrary threshold (16).

Tissue sampling. Animal use was approved by the San Diego VA Subcommittee on animal studies, protocol 02-037. For localization of cathelicidin-related antimicrobial peptide (CRAMP; mouse cathelicidin homologue) by immunohistochemistry, mammary glands were dissected from lactating C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) housed in a pathogen-free barrier facility. After mice were killed by CO_2 inhalation, tissue was immediately embedded in OCT compound. Mammary glands were microdissected for extraction and immunostaining. All specimens were stored at −80°C until use. We did not examine nonlactating mouse mammary glands because of difficulty in isolating sufficient tissue.

Immunohistochemistry. For CRAMP protein, rabbit polyclonal antibodies to CRAMP were used as previously described (18). Tissue Sections (10 μm) were immersed in PBS after 4% paraformaldehyde fixation for 10 min, and endogenous peroxidase activity was blocked with a 30-min incubation in 0.3%

H_2O_2 in methanol. Sections were blocked with 2% goat serum in PBS for 30 min and incubated with primary antibody, rabbit polyclonal antibodies to CRAMP (0.8 μg/mL) in PBS, 3% BSA for overnight at 4°C. Signal was detected with Vectastain ABC Elite Rabbit kit (Vector Laboratories, Burlingame, CA) and processed using an ABC kit (Vector Laboratories). As a negative control, nonimmunized rabbit IgG was diluted with PBS that contained 3% BSA to the same protein concentration as the primary antibody. Sections were incubated in 0.02% diaminobenzidine with 0.05% H_2O_2 in PBS for 2 min and counterstained with Mayer's hematoxylin for 10 s. The specificity of the primary antibody reaction was confirmed in separate experiments by absorption of the anti-CRAMP with excess amounts of synthetic peptide.

Immunoprecipitation and Western blot analysis. Rabbit polyclonal antibodies to LL-37 used for immunostaining were derived against full-length LL-37, whose sequence is LLGDFFRKSKEKIGKEFKRIVQRIKDFL-RNLVPRTES (QED Bioscience Inc., San Diego, CA). Stock concentration of antibody was 0.73 mg/mL. Chicken polyclonal antibodies to LL-37 mature form and cathelin domain were also used for reprobing, as previously described (19).

For immunoprecipitation, 100 μL of human milk was mixed with 20 μL of rabbit polyclonal antibodies to LL-37 and incubated for 60 min at 4°C, and then 20 μL of protein A-Agarose (Roche, Indianapolis, IN) was added and incubated overnight at 4°C. After centrifuging at $1000 \times g$ for 2 min, the supernatant was removed and the pellet was washed three times with 120 μL of PBS (pH 6.5). Beads were then resuspended in 50 μL of Tris glycine sample buffer with 10% β-mercaptoethanol, denatured at 85°C for 10 min, then centrifuged at $1500 \times g$ for 5 min. SDS-PAGE was on a 4–20% Tris-glycine PAGE system (Express gels; ISC BioExpress, Kaysville, VT). Gels were transferred to nitrocellulose membranes (OSMONICS, Westborough, MA), blocked [0.1% TTBS: 5% nonfat milk in 0.1% Tween 20/Tris-buffered saline; TBS: 150 mM of NaCl, 10 mM of Tris base (pH 7.4)] for 60 min at room temperature, and probed with rabbit polyclonal antibodies to LL-37 (1:5000 in the blocking solution) overnight at 4°C. After the nitrocellulose membranes were washed three times with 0.1% TTBS, horseradish peroxidase–labeled goat polyclonal antibody to rabbit IgG (1:5000 in the blocking solution) was incubated with the nitrocellulose membranes for 60 min at room temperature. Signal was developed in ECL solution (Western Lightning Chemiluminescence Reagents Plus; New Lifescience Products, Boston, MA) and exposed for 60 s to x-ray film (Kodak).

For confirmation of the identity of the band detected by Western blot, filters were stripped of antibody in [62.5 mM of Tris-HCl (pH 6.8), 2% SDS, and 100 mM of β-mercaptoethanol] at 50°C for 30 min, then reacted with a chicken antibody to cathelin (1:10000) and horseradish peroxidase–goat antibody to chicken IgY (1:10000; Aves Labs, Tigard, OR).

Measurement of LL-37 concentration in human milk. To estimate the concentration of LL-37, we performed quantitative dot-blot analysis. Ten microliters of human milk sample (day 10) was compared with a standard curve of various concentrations of synthetic peptide applied onto nitrocellulose filters (14). LL-37 was detected as described for Western blot above.

Antimicrobial assay in human milk. The antimicrobial activity of LL-37 was evaluated in an ionic environment similar to human milk by colony-forming unit assays performed with *Staphylococcus aureus* (clinical isolate), group A *Streptococcus* (GAS; NZ131), and enteroinvasive *Escherichia coli* O29 as described (20). For these experiments, proteins in separate aliquots of human milk collected at day 10 were denatured. Aliquots were autoclaved at 131°C and then centrifuged at $10,000 \times g$ for 15 min. Afterward, the supernatant fluids were collected. This process was repeated five times until the color of the milk preparation was clear light yellow [autoclaved milk supernatant (AMS)]. Before analysis, concentration of the bacteria in culture was determined directly by plating different bacterial dilutions (at A_{600}, 1.0 corresponded to 3.5×10^9/mL for *S. aureus*, 2.5×10^8 for GAS, and 3.5×10^8 for *E. coli*). Bacteria were washed twice with 10 mM of phosphate buffer and diluted to a concentration of 2×10^6 cells/mL (*S. aureus* and GAS) or 2×10^5 cells/mL (*E. coli*) in AMS and incubated for 2 h at 37°C with various concentrations of LL-37 in 50 μL of 1× AMS or 0.1× AMS using wells of a 96-well round-bottom tissue culture plate (Costar 3799, Corning). Bacteria in AMS were then diluted from $10×$ to $10^{5×}$, and 10 μL of each of those solutions was plated in triplicate on Todd Hewitt broth plates for direct determination of colony-forming units.

RESULTS

Human cathelicidin hCAP18/LL-37 and hBD-1 mRNA are present in human milk. For determining whether transcripts for the antimicrobial peptides hCAP18/LL-37, hBD-1, and hBD-2 were present in cellular elements of human milk, reverse transcriptase–PCR (RT-PCR) was performed on cells that were derived from human breast milk collected at $10-60$ d after parturition (Fig. 1). In these preparations, LL-37 and hBD-1 were detectable, but hBD-2 was not.

Next, for quantitatively evaluating and confirm the expression of LL-37 seen in Fig. 1, LL-37 mRNA levels were examined by quantitative real-time RT-PCR. Compared with expression at day 10, the abundance of LL-37 in day 30 and day 60 human milk cells showed a 12- and 15-fold increase. hBD-1 mRNA expression in day 30 was ~5-fold higher compared with the level at day 10 (Fig. 2).

Cathelicidin proteins are localized in mouse mammary glands and secreted in human milk. To further explore and define cathelicidin protein expression in the mammary gland, we examined breast tissue from lactating mice for CRAMP by immunohistochemistry and evaluated human breast milk peptides by Western blot. Abundant CRAMP was detected in the lactating lobular units of the mouse (Fig. 3*a*). The signal was diffusely located in the cytoplasm of lobuloacinar and ductules, but not in ducts (Fig. 3*c*). No signal was detected in tissues using IgG controls. (Fig. 3*b* and *d*).

The detection of cathelicidin protein in the murine breast tissue cell suggested that this antimicrobial peptide might also be present and secreted in active form in human milk. To study this, human milk (day 10) was evaluated by Western blot and quantified by dot-blot analyses with two antibodies independently derived against distinct domains of the cathelicidin proprotein: anti-LL-37 and anti–cathelin domain. After immunoprecipitation, LL-37 was detected in human milk (Fig. 4*a*). In specific immunoprecipitates, a band at ~4.5 kD correspond-

Figure 2. Measurement of hCAP18/LL-37 and hBD-1 mRNA in human milk cells by real-time PCR. For comparing the relative expression of hCAP18/LL-37 or hBD-1 mRNA in human milk cells over time after delivery, quantitative real-time RT-PCR was performed. Transcript abundance at days 10, 15, 30, and 60 are shown normalized to the relative abundance of each gene product at day 10. hBD1 (▨) demonstrated a 6-fold increase at day 30 relative to day 10. hCAP18/LL37 (■) increased 12-fold by day 30, and expression persisted up to day 60. Data are triplicate measurements from three individuals $\pm$ SEM and are representative of two experiments.
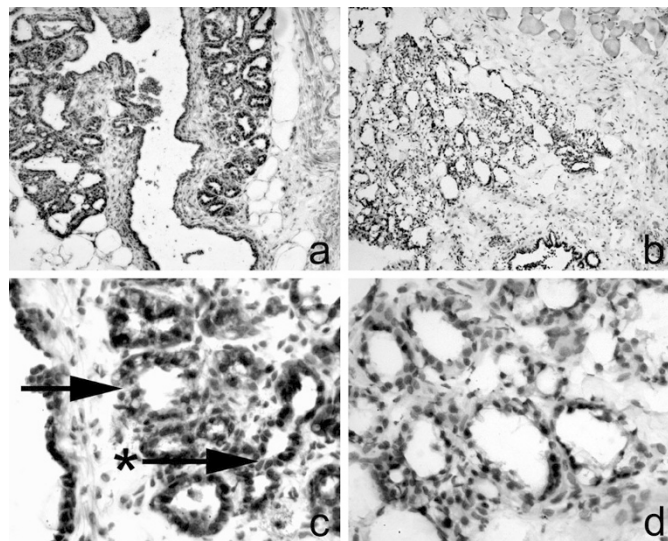
Figure 3. Mouse cathelicidin expression in mammary glands. For defining cathelicidin expression in breast tissue, mammary glands from lactating mice were examined for CRAMP expression by immunohistochemistry. (*a* and *c*) Tissues stained for CRAMP. (*b* and *d*) Negative controls. Abundant CRAMP was detected in the lactating lobular unit (*a*). The signal was diffusely located in the cytoplasm of lobuloacinar (arrow) and ductules (arrow with asterisk; *c*). Rabbit IgG used for negative control showed no detectable signal in these tissues (*b* and *d*). Magnification: ×100 in *a* and *b*; ×400 in *c* and *d*.
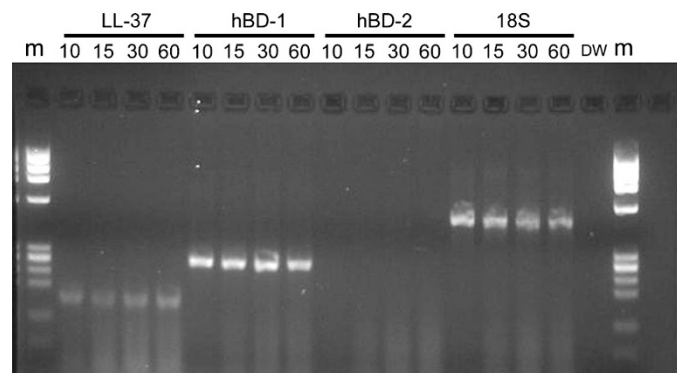

Figure 1. hCAP18/LL-37 and hBD-1 mRNA is detectable in human milk cells by RT-PCR. One microgram of total RNA was used for each RT reaction. The human milk specimens analyzed were obtained at 10, 15, 30, and 60 d after delivery. hCAP18/LL-37 and hBD-1 mRNA was detected in all samples but not hBD-2. The expected sizes of PCR products were 272, 254, and 173 bp for hBD-1, hBD-2, and LL-37, respectively. Internal 18S was amplified as a loading control, and distilled water (DW) was used as a negative control. The ladder (m) is 10 μL of phi-X 174/*Hae*III size marker. Data shown are from one sample preparation from one individual and are representative of four separate sample preparations from each of three individuals.
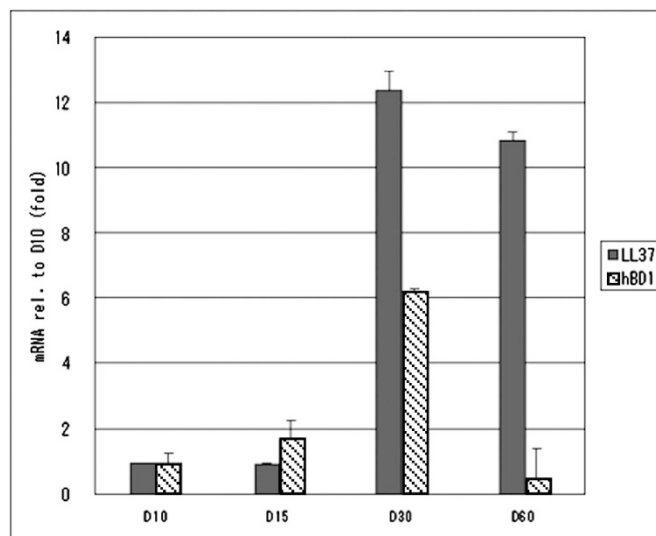
ing to the mature processed form of LL-37 was detected. Large molecular weight immunoreactive material was seen predominantly in unbound washed material from immunoprecipitates, possibly due to nonspecific reactivity of rabbit IgG with human milk proteins, aggregation of LL-37 in a form not precipitable, or unprocessed and aggregated hCAP18/LL-37 also in a form not precipitable. To confirm a lack of the unprocessed precur-
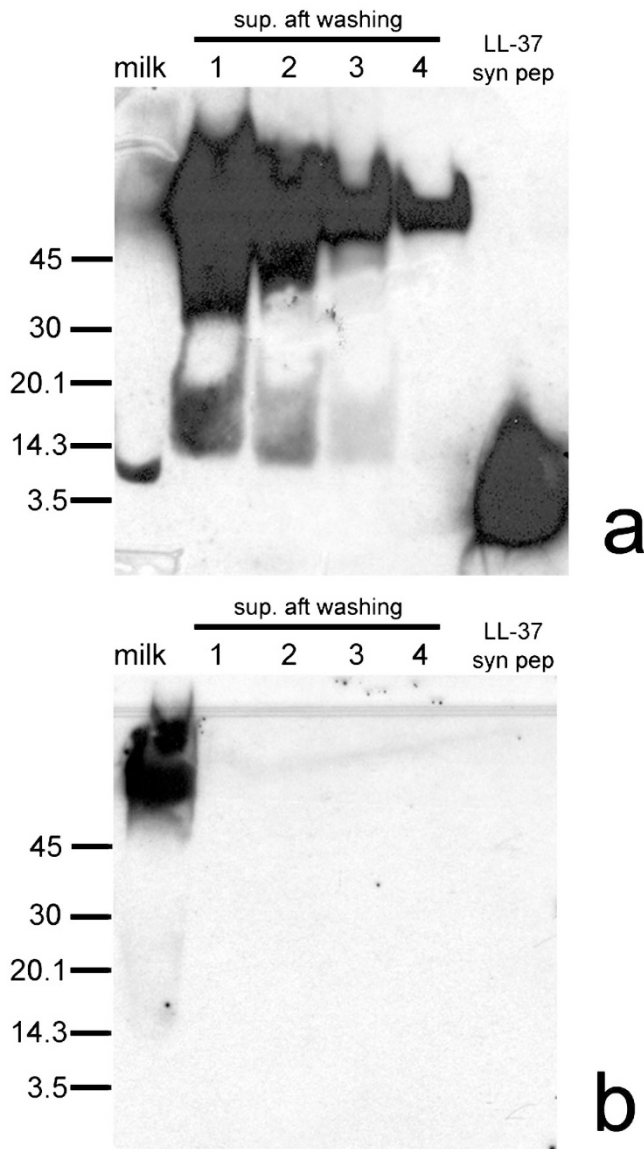
Figure 4. Human milk contains the mature cathelicidin peptide LL-37. Human breast milk collected at day 10 was evaluated by immunoprecipitation and Western blot with antibody specific to LL-37 (*a*). The far left lane (milk) shows single band by Western blot migrating at a size consistent with the expected 4.7-kD mature form of LL-37 and similar to 100 pmol of LL-37 synthetic peptide loaded in the far right lane (LL-37 syn pep). The broad band seen in the synthetic LL-37 lane is due to the sample's signal intensity, not variability in peptide size. Lanes 1, 2, 3, and 4 show immunoreactive material successively washed but not immunoprecipitated, representing aggregates of LL-37 and milk proteins, unprocessed hCAP18/LL37, or nonspecific binding. The nitrocellulose membrane was stripped and reprobed with antibody against the cathelin immature domain present only in unprocessed hCAP18/LL37 (*b*). No specific band was detected at the expected size of 18 kD.

sor cathelicidin in human milk, we stripped the nitrocellulose membranes and reprobed them with chicken antibody specifically against the cathelin immature domain. No specific band was detected at the expected size of 18 kD for hCAP18/LL-37 (Fig. 4*b*), although reactivity was detectable at high molecular weight in the immunoprecipitate. To quantitatively evaluate the concentration of LL-37 in human milk, we performed dot-blot analysis. LL-37 abundance was ~1 μmol/21.7 μg of total milk protein (milk protein concentration 764 μg/mL) or 35 μM.

LL-37 is a functional antimicrobial peptide in human milk. To evaluate whether LL-37 has antimicrobial activity in the ionic environment of the human milk, we performed bactericidal assays in an AMS solution. GAS, *S. aureus*, or *E. coli* was incubated for 2 h at 37°C in AMS (Fig. 5). GAS, *S. aureus*, and *E. coli* were first incubated in AMS without addition of synthetic LL-37 peptide to evaluate whether AMS alone was antimicrobial. After 2 h of incubation, 75% of GAS, 98% of *S. aureus*, and 100% of *E. coli* survived in AMS but did not proliferate. Because existing antibody to LL-37 does not neutralize bioactivity, it was not feasible to directly evaluate the contribution of LL-37 to the antimicrobial activity observed in AMS. Furthermore, the lack of bacterial growth in this solution prevented determination of inhibitory activity. Therefore, the effect of supplemental addition of synthetic LL-37 on direct bacterial killing was tested (Fig. 5*a*). LL-37 was an effective antimicrobial in the human milk environment but demonstrated increased cytotoxic activity against test microbes when AMS was diluted. A total of 32 μM of LL-37 killed 77% of GAS in 1× AMS and 100% in 0.1× AMS (Fig. 5*b*). Against *S. aureus*, 32 μM of LL-37 killed 40% in 1× AMS and 99% in 0.1× AMS (Fig. 5*c*). Against *E. coli*, 32 μM of LL-37 killed 17% in 1× AMS and 100% in 0.1× AMS (Fig. 5*d*).
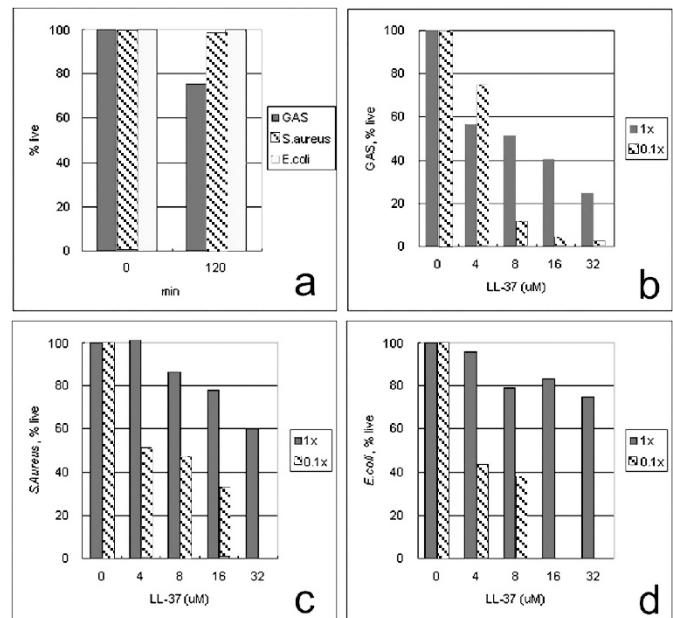

Figure 5. LL-37 synthetic peptide kills *E. coli*, *S. aureus*, and GAS in human milk solutions. The antimicrobial activity of synthetic LL-37 was evaluated in a heat-denatured solution prepared from human milk (AMS). Bacteria did not proliferate, and AMS had weak antimicrobial activity alone; thus, determination of the minimal inhibitory concentration (MIC) was not possible in this solution (*a*). For determining minimal bactericidal concentrations (MBC), LL-37 at concentrations between 0 and 32 μM was incubated for 2 h with GAS (*b*), *S. aureus* (*c*), and *E. coli* (*d*) in AMS(1×) or AMS diluted with water 10-fold (0.1×). Bactericidal activity was observed for all organisms but maximal in diluted AMS. Values are percentage of living bacteria (% live) compared with the control (no synthetic peptide). Data shown are representative of triplicate determinations from three independent experiments.

DISCUSSION

Peptides with antimicrobial activity are found throughout nature (1). First isolated from mammalian granulocytes (21,22), peptides such as the defensins and cathelicidins are present in intracellular granules, where they can facilitate microbial killing by phagocytic cells such as neutrophils and mast cells (6,23). The ability of these antibiotic gene products to inhibit microbial proliferation is also associated with the immune defense strategy of epithelia from organs such as skin, lung, gut, and kidney. At the epithelial interface, production of antimicrobial peptides is thought to provide an important first line of defense against infection. In this study, we investigated whether the cathelicidin antimicrobial peptide LL-37 is present in human milk and the murine gene homologue CRAMP is expressed in lactating mouse mammary tissue. Results show that human cathelicidin hCAP18/LL-37 mRNA can be detected in cells shed into human milk for at least 60 d after parturition. This expression is coincident with the presence of hBD-1, a distinct mammalian antimicrobial peptide previously described in mammary epithelia (13,24). Importantly, the hCAP18/LL-37 gene product was also demonstrated in soluble form in human milk and present in its activated form as the peptide LL-37. This peptide was directly shown to have antimicrobial activity in aqueous solution derived from human milk. These observations suggest that production of cathelicidins by mammary tissue may be an important component of innate immune defense during lactation.

The detection of cathelicidin mRNA in the cellular elements of human milk was based on a sensitive RT-PCR approach. Human milk leukocytes, mammary epithelial cells, or epithelial cells from the surface of the breast could conceivably contribute to the detection of transcripts by this technique. A previous study of human milk epithelial cells showed that hBD-1 but not hBD-2 mRNA is present in these cells (24). Our findings are consistent with these results. Because hBD-2 was not detected but is likely present in the surface keratinocytes of breast skin (25), it is unlikely that the origin of the detectable transcript is from surface skin contamination. Previously, hBD-2 mRNA was found in breast tissue by RNA dot-blot and *in situ* hybridization (26). However, as in a previous study of hBD-1 expression in human milk cells (24), we failed to detect the presence of hBD-2 transcripts. This apparent conflict may be due to instability of hBD-2 message. In addition, it is possible that the mammary expression of antimicrobial peptides occurs at both a constitutive and inducible level. Under the present conditions, it seems that hBD-1 and LL-37 are each constitutive products of the lactating mammary gland. Other epithelia show a large induction of cathelicidins and defensins in response to injury or bacterial challenge (18), a phenomenon that may have contributed to expression of hBD-2 in previous studies.

Lactating mice were used as an animal model to further explore the expression of cathelicidins in breast tissue. The mouse cathelicidin CRAMP is a close homologue of human hCAP18/LL-37 and has been found to correlate with human expression patterns in previous investigations (16,18,19). Furthermore, the availability of a knockout mouse deficient in

CRAMP has enabled investigation of the biologic significance of cathelicidin expression *in vivo* (23). In the present study, CRAMP expression was found in the cytoplasm of lobuloacinar and ductule cells. This is distinct from previous reports of the expression of hBD-1 protein and hBD-2 mRNA in ductule epithelium. Unique expression patterns for defensins and cathelicidins are consistent with that observed in other tissues and may suggest distinct stimuli and targets for their gene products. In addition, cathelicidins and defensins have synergistic action as antimicrobials (27). Observation that both are expressed in the lactating mammary gland enables co-localization and the potential for enhanced immune defense.

In human milk, cathelicidin was detected exclusively in its activated peptide form. When first synthesized as a precursor of ~18 kD, the hCAP18/LL-37 protein is thought to be inactive. Processing of full-length precursor cathelicidins has been associated with several different serum proteases, including elastase and proteinase 3 (28). After processing, the N-terminal cathelin protein has activity as both an antimicrobial and a protease inhibitor (29). This precursor protein was not detectable in human milk, although techniques used for detection of the precursor protein were not optimized in this system. Further work is required to confirm that unprocessed or the cathelin protein alone is not secreted from mammary tissue. However, LL-37 appeared relatively abundantly in human milk at concentrations approaching 32 μM. At this concentration, LL-37 synthetic peptide is a potent antimicrobial against a wide range of microbes. However, antimicrobial activity reported for naturally occurring peptides is highly dependent on environmental conditions. Factors such as pH, sodium chloride concentration, and binding to other macromolecules in the soluble solution are critical determinants of the ability of antimicrobial peptides to interact with the microbial target membrane (30,31). For assessing antimicrobial activity in the ionic environment of milk, a heat-denatured human milk solution was produced and antimicrobial activity against *S. aureus* or *E. coli* was determined. Our results suggest that LL-37 has direct bactericidal activity on these bacteria in human milk solution. Actual activity *in vivo* may be most relevant for bacterial growth inhibition or further augmented by the synergistic presence of other natural antimicrobial molecules in human milk such as IgA, lactoferrin, and casein-k (12). Molecules such as lactoferrin are inactivated by heat sterilization (32), thus underestimating the potential antimicrobial effect of LL-37 observed here. Inhibitory activity from native LL-37, hBD-1, or other heat-resistant molecules in AMS may also have contributed to the growth inhibitory activity observed for AMS alone. Further work to specifically evaluate human milk in the absence of these molecules will be required to definitively identify their contribution to milk's antimicrobial properties. At present, the biologic activity of cathelicidins *in vivo* remains uncertain.

The presence of antimicrobial molecules in human milk may be important for protection of the mammary gland against infection and development of mastitis, protection of milk from microbial proliferation after secretion, or protection of the infant as a result of ingestion of naturally occurring antibiotics (33). The ability of cathelicidins and defensins to directly

confer protection against bacterial colonization of epithelial surfaces has been shown in gut, lung, and skin (34). Therefore, it is reasonable to propose that these molecules may also provide a protection against mastitis. Possible protection to the neonate was suggested in studies of neonatal rat pups that were reared by artificial formula feeding. These pups have an increase in gut *Enterobacteriaceae* compared with those that are fed breast milk (35). This increase in colonization and bacterial translocation may be a consequence of a direct antimicrobial action of milk. Identification of additional candidate genes such as cathelicidins in breast milk provides further information for study of this important system. Of interest, the expression of antimicrobial peptides in milk is a prolonged phenomenon that extends long into the postnatal period. This is in marked contrast to other presumed protective components, such as immunoglobulin, that decrease after birth or the increase in lysozyme secretion seen over time (36,37). A constant concentration of cathelicidins as lactation volume increases suggests that total production of antimicrobial peptides increases to meet milk production. These data support the importance of antimicrobial peptides to immune defense and suggest that further research is required to delineate the biologic role of small cationic peptides in the protection of the mammary gland, the infant, or both.

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