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Expression of a β-Defensin mRNA, Lingual Antimicrobial Peptide, in Bovine Mammary Epithelial Tissue Is Induced by Mastitis

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The expression of a β-defensin, the lingual antimicrobial peptide (LAP), in response to mastitis was investigated by real-time PCR of RNA from mastitic and control udder quarters. There was a positive relationship between somatic cell count in milk and LAP expression. In situ hybridization showed that LAP mRNA was expressed in epithelial cells of mastitic tissue. These results suggest that LAP plays a role in the innate immune response to mastitis.

Bovine mastitis, caused by bacterial or fungal pathogens and resulting in an elevated level of somatic cells in milk, is one of the most costly dairy-based diseases worldwide, impairing both milk yield and processing quality (7, 8). Phagocytic cells constitute a major part of the defense mechanism of the bovine mammary gland against mastitis-causing pathogens (4, 12). However, little is known about the antimicrobial activity of other compounds expressed within the bovine mammary gland.

Defensins contribute to the innate immune response and are effective against a variety of microorganisms (6). Expression of β-defensin RNA varies in epithelial cells, with the highest levels being in those tissues that are constantly exposed to, and colonized by, microorganisms (3, 15). Recently, the presence of Toll-like receptors 2 and 4 and a gene encoding β-neutrophil defensin 5 were reported to be up-regulated in response to mastitis within the bovine mammary gland (2).

The lingual antimicrobial peptide (LAP) is a member of the β-defensin family and was first isolated from inflamed bovine tongue epithelium (13). Subsequent investigations found widespread LAP expression in infected bovine intestinal and respiratory tissue (15).

Here we examined the expression of LAP in the bovine mammary gland to determine if this β-defensin plays a role in the innate immune defense during a mammary infection.

Mammary tissue was obtained from eight cows with mastitis (Table 1). Three cows had naturally occurring infections (cows 1 to 3). The infection status was determined by milk somatic cell count (SCC) analysis and bacteriological analysis (14). In cows 4 to 8, mastitis was induced with a wild-type strain of *Streptococcus uberis* (isolated from a cow with clinical mastitis) by infusing 1,000 to 1,500 CFU via the teats into two quarters. The cows were observed for clinical signs of mastitis, evident as swelling and protein aggregates in the milk. Alveolar, cisternal, and peripheral tissue was taken postmortem from the infected quarter and a control noninfused quarter within 24 h following the identification of clinical mastitis (Table 1). The peripheral region was defined as 1 to 2 cm below the connective tissue layer in the outer regions of the mammary gland. Liver and white blood cell samples were taken from healthy cows free of mastitis.

Total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, Calif.) and converted to cDNA by using the SuperScript first-strand synthesis system for real-time PCR (Invitrogen). Approximately 5 ng of the cDNA synthesis reaction mixture was used for quantitative PCR amplification. Primers were designed for the LAP coding region (114 bp) and repeat region 4 of bovine polyubiquitin (201 bp) (Table 2). Amplification, detection, and real-time analysis were performed by using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif.). SYBR Green I (Applied Biosystems) was used for detection of the amplified product. The thermal cycling program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, and 78°C for 10 s. Following each real-time experiment, dissociation curves were run to ensure that there was only one product and that no primer dimers were present.

There was a significant ($P < 0.001$) positive relationship between the log-normalized amounts for SCCs and LAP expression in all three regions of the mammary gland (Fig. 1). This relationship was calculated by fitting a common slope of the graph for log LAP expression on log SCCs (Fig. 1) from the mastitic and control glands of the eight cows (the specification of cows is a random effect) by using REML (GenStat for Windows, 2002, version 6.1, 6th ed.; VSN International Ltd., Oxford, United Kingdom). Increasing the SCC 10-fold, which corresponds to log(SCC) increasing by 1, is associated with an increase of 0.78 ± 0.098 in log(LAP, alveolar) or a 6-fold increase in LAP (95% confidence interval, 3.8 to 9.4). The increase in log(LAP, cisternal) was 0.66 ± 0.083, indicating a 4.5-fold increase in LAP (95% confidence interval, 3.1 to 6.7), and the increase in log(LAP, peripheral) was 0.68 ± 0.087, indicating a 4.8-

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TABLE 1. SCCs and bacteriology results for individual quarters

Cow	Infection status	Quarter sampled[a]	SCC (10^3 cells/ml)	Bacteriology comment
1	Noninfected	RF	62	No specific growth
	Clinical infection	RB	19,430	Heavy growth of *Staphylococcus* species
2	Subclinical infection	RF	895	Light growth of *Corynebacterium* species
	Clinical infection	RB	9,122	Moderate growth of *Corynebacterium* species
3	Clinical infection	RB	7,190	Moderate growth of *Staphylococcus aureus*
	Clinical infection	LB	22,379	Heavy growth of *Staphylococcus aureus*
4	Noninfected	LF	38	No specific growth
	Clinical infection	RB	16,147	Heavy growth of *Streptococcus uberis*
5	Noninfected	LB	30	No specific growth
	Clinical infection	RB	1,786	Heavy growth of *Streptococcus uberis*
6	Noninfected	RF	219	No specific growth
	Clinical infection	RB	13,865	Heavy growth of *Streptococcus uberis*
7	Noninfected	LB	116	No specific growth
	Clinical infection	RB	18,912	Heavy growth of *Streptococcus uberis*
8	Noninfected	RB	93	No specific growth
	Clinical infection	LF	17,297	Heavy growth of *Streptococcus uberis*

[a] LF, left front; LB, left back; RF, right front; RB, right back.

fold increase in LAP (95% confidence interval, 3.2 to 7.1). LAP gene expression was not detected in white blood cells or liver samples (data not shown).

In agreement with data from other diseased tissues (13, 15), LAP mRNA expression increased in infected tissue from all regions within the mammary gland. Exposure to lipopolysaccharides or inflammatory cytokines has also been shown to increase LAP mRNA expression in bovine tracheal epithelial cells (11). This increase in LAP mRNA was independent of the infecting organism. However, all the bacteria in this study were gram positive. We have not shown that LAP mRNA increases with a gram-negative infection, but it has been shown previously to exhibit antimicrobial activity against gram-negative bacteria (13), and some neutrophil defensins have antimicrobial activity against gram-negative organisms isolated from mastitic cows (1).

Infected and noninfected tissue from cows 2 and 3 were used for in situ hybridization to determine the cell-specific mRNA expression of LAP. Histological tissue samples were fixed and embedded in paramat wax (BDH Laboratory Supplies, Dorset, United Kingdom). Serial sections (7 μm thick) were cut and mounted onto Polysine-coated microscope slides (Biolab Scientific, Auckland, New Zealand). A 220-bp cDNA sequence was isolated by using real-time PCR with primers to conserved regions of β-defensins from lactating bovine mammary tissue. The forward primer sequence used for this isolation was 5′-TTCGGTACCGACAGCATGAGGCTCC, and the reverse primer sequence was 5′-TGACTCCGCATCCAGTCTGGCCACG (unpublished data). The isolated cDNA sequence was identical to the published bovine LAP nucleotide sequence (NCBI Entrez nucleotide database, accession number S76279). Sense and antisense ^{35}S-UTP (Amersham) riboprobes were prepared from linearized cDNA plasmids containing the LAP sequence and hybridized with mounted tissue sections in accordance with published methods (9).

Hybridization of the ^{35}S-labeled LAP antisense probe detected expression of LAP within the mastitis-affected epithelial cells. Abundant expression of LAP mRNA was located in the ductal linings of the teat and cistern of mastitic quarters (Fig. 2A to C), suggesting that LAP mRNA expression is induced at the site of infection, as these tissues would be the first exposed to invading microorganisms. These findings extend those of previous studies, which have shown the expression in teat and other ductal tissue of lactoferrin, a known antimicrobial component in milk (10). LAP mRNA expression was detected in epithelium from the cisternal, alveolar, and peripheral regions of infected tissues. In contrast, LAP mRNA expression was not detected in the noninfected tissue sections (data not shown).

LAP mRNA was detected in some secretory alveoli cells (Fig. 2D and E), but expression was limited to alveolar cells which displayed obvious signs of inflammation, such as the presence of neutrophils in the alveolar lumen or in regressed alveoli. No signal could be detected when these tissues were hybridized with the control LAP sense probe (Fig. 2F). No expression was detected in secretory tissue from noninfected quarters (data not shown). LAP is expressed within both the epithelial cells of ductal linings and the alveoli. This observation supports the real-time PCR data showing that the alveolar

TABLE 2. LAP and ubiquitin primers used for real-time PCR and PCR results

Gene[a]	Primer sequence (5′→3′)	Position in coding sequence	PCR product length (bp)	EMBL accession no.[a]
LAP forward	GAAATTCTCAAAGCTGCCGTA	100	114	S76279
LAP reverse	TCCTCCTGCAGCATTTTACTT	193		
Ubiquitin forward	GGCAAGACCATCACCCTGGAA	798	201	Z18245
Ubiquitin reverse	GCCACCCCTCAGACGAAGGA	998		

[a] GenBank accession number of the published bovine nucleic acid sequence.

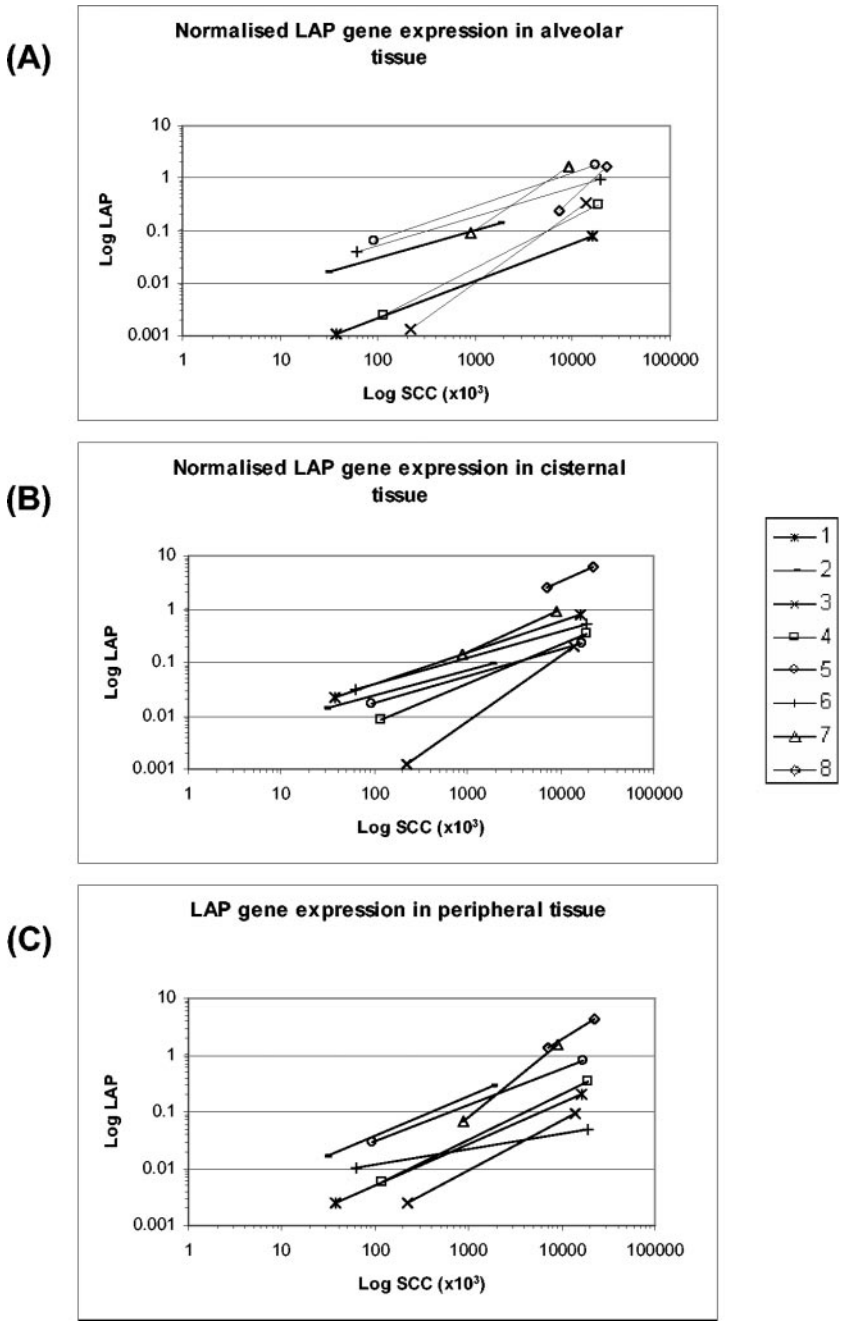

FIG. 1. Levels of LAP gene expression in three regions of the bovine mammary gland in each of the eight cows. Normalized amounts of LAP mRNA in individual udder quarters for all cows are plotted. Each point is represented by a symbol with lines connecting noninfected and infected tissue samples from each cow. The slope of the line represents the correlation between log LAP gene expression and log SCCs. (A) Alveolar tissue; (B) cisternal tissue; (C) peripheral tissue.

tissue has the highest level of LAP in the mammary gland. The LAP cDNA probe did not hybridize to leukocytes, therefore confirming probe specificity under our experimental conditions. This finding suggests that LAP transcription is induced following infection, as it was not present at detectable levels in lactating alveolar cells or alveoli without leukocytes in their lumens. Expression of LAP mRNA within the secretory epithelium suggests the release of LAP onto the epithelial surface and possibly into milk, similar to what occurs with β-defensin-1 in human milk (5).

In this study, we have demonstrated the inducible mammary expression of LAP in mammary epithelial cells in response to mastitis. These results indicate a role for LAP in the immune response against mastitis in the cow.

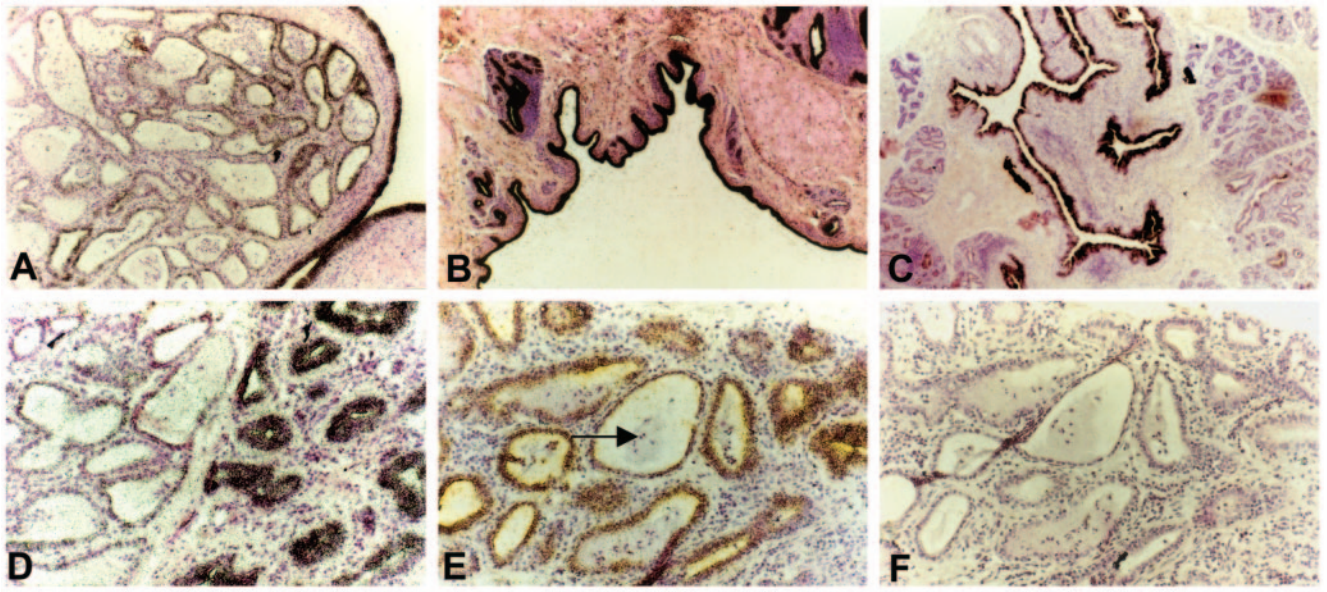

FIG. 2. In situ hybridization of the LAP ^{35}S-labeled probe to infected tissue sections of the bovine mammary gland. The tissue sections were probed with the ^{35}S-labeled LAP sense and antisense probes. Hybridized sections were exposed for 40 days under nuclear emulsion at 4°C. High-intensity expression is demonstrated as dense black labeling by silver grains over the cells. (A) Cow 3, infected cisternal tissue. Magnification, ×100. (B) Cow 2, infected teat epithelium. Magnification, ×100. (C) Cow 2, subclinical infection (low SCC) cisternal tissue. Magnification, ×40. (D) Cow 3, infected cisternal-alveolar junction. Magnification, ×200. (E) Cow 3, subclinical alveolar tissue. No signal is detected in the white blood cells (arrow) within the alveoli. (F) Control serial tissue of the sample from panel E probed with the LAP sense probe. Magnification, ×200.

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