Imbalance Between Lipoxin A₄ and Leukotriene B₄ in Chronic Mastitis-Affected Cows

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ABSTRACT

Persistent accumulation of inflammatory cells in the udder, with neutrophils being the predominant cell type, is a characteristic feature of chronic mastitis in dairy cows. Leukotriene (LT) B₄ is a potent chemotactic agent, known to induce recruitment and accumulation of neutrophils in the bovine mammary gland. The LTB₄-stimulated neutrophil functional responses are closely opposed by lipoxin (LX) A₄, which promotes the resolution of inflammation. We thus hypothesized that the chronic inflammation of the udder could be associated with an unfavorable ratio between these two eicosanoids and that the persistence of neutrophil accumulation could be due to an increase in LTB₄ synthesis and/or an impaired LXA₄ production. In an attempt to verify this hypothesis, we first measured LXA₄, LTB₄, and their ratio in the milk of healthy and acute and chronic mastitis-affected quarters. Next, we studied the relationships between these variables and the degree of udder inflammation as assessed by somatic cell count measurement. The LTB₄ concentration was low in healthy quarters, drastically increased in acute mastitis, and reached intermediate levels in chronic mastitis-affected quarters. However, whereas LXA₄ concentration was highly increased in acute mastitis, healthy and chronic quarters had similarly low values. The LXA₄:LTB₄ ratio was thus significantly lower in chronic mastitis-affected cows. The LTB₄ concentrations measured in chronic quarters were highly correlated to somatic cell count and to milk neutrophil and macrophage numbers. A weaker correlation was observed between LXA₄ and these variables. For both eicosanoids, the highest correlation was observed with the number of neutrophils. These results show the existence of an LXA₄:LTB₄ imbalance in chronic mastitis-affected

cows because of low LXA₄ concentrations. Further studies are needed to determine whether administration of LX or stable analogs could have therapeutic potential in the control of chronic bovine mastitis.

(Key words: chronic mastitis, imbalance, leukotriene B₄, lipoxin A₄)

Abbreviation key: ATL = aspirin-triggered lipoxin, LO = lipoxygenase, LT = leukotriene, LX = lipoxin, NF- κ B = nuclear factor κ B, PMN = polymorphonuclear leukocytes, QMS = quarter milk sample, TNF = tumor necrosis factor.

INTRODUCTION

Mastitis is an inflammatory reaction within the udder, usually resulting from bacterial infection (Harmon, 1994). Chronic subclinical mastitis is frequent and causes major economic losses in dairy cattle due to damage to alveolar tissues, reduction in milk yield, and deterioration in milk composition (Gudding et al., 1984). A better understanding of the mechanisms by which inflammation persists in the udder therefore represents a great challenge.

The most common bacteria associated with subclinical infections are contagious mastitis pathogens, such as *Staphylococcus aureus* and *Streptococcus agalactiae*, for which the major reservoir is the infected udder (Eberhart et al., 1987). When bacteria enter the mammary gland, a drastic increase in milk SCC occurs, with polymorphonuclear (PMN) neutrophil being the predominant cell type (Paae et al., 1979; Burvenich et al., 1994). Migration of neutrophils to the site of inflammation is one of the most important defense mechanisms. Neutrophils need to be recruited promptly and in sufficient numbers to avoid excessive growth of bacteria and death of the animal (Burvenich et al., 1994). However, neutrophils can also be detrimental to tissues by releasing intracellular products that exacerbate the inflammatory process resulting in chronic inflammation, thus impairing the function of the organ (Harmon, 1994).

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Neutrophil recruitment and sequestration to sites of inflammation is initiated by proinflammatory mediators, among which leukotriene (LT) B₄ is considered to be important (Ford-Hutchinson et al., 1980; Weissmann et al., 1980; Samuelsson, 1983). Leukotriene B₄ is formed by the breakdown of arachidonic acid, via the 5-lipoxygenase (LO) enzyme pathway, giving LTA₄ then converted to LTB₄. Rose et al. (1989) revealed LTB₄ in the milk of *Klebsiella pneumoniae*-inoculated quarters and suggested that neutrophils initially attracted to a mastitic quarter may be stimulated to release LTB₄, which may in turn increase neutrophil migration and prolong neutrophil survival in mammary tissue. This leads to the assumption that persistence of LTB₄ production could be of particular importance in the pathogenesis of chronic inflammation in mastitis.

The natural resolution of an inflammatory response is fundamental to health. It depends on multiple processes that include the generation of endogenous "breaking signals" that facilitate a return to the pre-morbid state (McMahon et al., 2001). Recent results provide evidence for a new appreciation that certain LO-derived eicosanoids possess potent "anti-PMN" actions and anti-inflammatory properties (Serhan et al., 1996; Diamond et al., 1999; Munger et al., 1999). In particular, lipoxins (LX), such as LXA₄ and aspirin-triggered LX (ATL), are arachidonic acid-derived bioactive lipids as a result of their exposure to the unique combinations of lipoxygenases that occur during specific heterotypic cell-cell interactions, such those occurring during inflammation (e.g., epithelial-neutrophil interactions). Mobilization of a LX biosynthetic circuit occurs, for example, when infiltrating PMN (which express 5-LO) interact with tissue resident cells (which express 15-LO) in inflamed target organs (Fierro and Serhan, 2001). These molecules appear to play an important role in downregulating neutrophil responses in inflammation (Serhan, 1997). In vitro studies have shown that LXA₄, ATL, and stable synthetic LX analogs inhibit PMN chemotaxis in response to LTB₄ (Lee et al., 1989; 1991). In experimental models in vivo, LXA₄ inhibits LTB₄-induced neutrophil margination and diapedesis in the hamster cheek pouch (Hedqvist et al., 1989) and in the mouse ear model of inflammation (Takano et al., 1997). Moreover, injection of a stable synthetic mimetic of ATL into murine dorsal air pouches inhibits leukocyte (neutrophils, eosinophils, basophils, and mononuclear cells) recruitment in response to tumor necrosis factor (TNF)- α (Hachicha et al., 1999), which stimulates the release of IL-8, a potent chemoattractant for neutrophils (Baggiolini et al., 1989; Persson et al., 1993). Furthermore, LX significantly enhances phagocytosis

of apoptotic PMN by human monocyte-derived macrophage in vitro, which suggests that LX can promote the clearance of apoptotic leukocytes by macrophages at the inflammatory site (Godson et al., 2000).

Knowing that LXA₄ is an anti-inflammatory eicosanoid that is closely opposed to LTB₄, a potent chemotactic eicosanoid, we hypothesized that persistent accumulation of neutrophils in chronic mastitis could be due, at least in part, to an increase in LTB₄ synthesis and/or impaired LXA₄ production in the udder. In an attempt to verify this hypothesis, we first measured LXA₄, LTB₄, and their ratio in milk of healthy, acute, and chronic mastitis-affected quarters. Next, we studied the relationships between these variables and the degree of udder inflammation as assessed by SCC measurement.

MATERIALS AND METHODS

Animals

Twenty-five healthy, 8 acute, and 40 chronic mastitis-affected Holstein-Friesian cows were used in this study. Healthy cows had a low SCC ($<10^5$ cells/ml) and were free of any clinical sign of mastitis. Acute mastitis-affected cows had high SCC ($>10^7$ cells/ml) and exhibited clinical signs of illness. Neither healthy nor acute mastitis-affected cows had any earlier record of udder disease as measured by clinical symptoms and monthly SCC recordings. Chronic mastitis-affected cows had persistently increased SCC (at least 3 consecutive times $>4 \times 10^5$ cells/ml), as determined by monthly SCC measurements, but were devoid of any clinical sign of the disease. In order to enter in the study, the chronic quarter SCC had to be $>4 \times 10^5$ cells/ml at the sampling time. All the cows were between 2 and 8 yr old, had a lactation number ranging from 1 to 6, and were lactating for 3 to 8 mo. Experimental cows did not receive any treatment during the month preceding the experiments.

Sterile Milk Sampling

Quarter milk samples (QMS), taken from one quarter, were collected prior to milking using sampling procedures recommended by the National Mastitis Council (1999). Teat ends were vigorously disinfected with a solution containing 70% alcohol. Approximately 10 ml of milk were collected into sterile vials. Samples were placed in freezer packs and delivered immediately to the mastitis diagnosis laboratory (Centres de Prévention et de Guidance Vétérinaire, Loncin, Belgium). Another 50-ml vial was collected for total and differential cell counts and LXA₄ and LTB₄ analyses.

Microbiological Analysis

Each QMS was incubated overnight at 37°C. Afterwards, aliquots (10 μ l) of each QMS were spread on sheep blood agar, McKonkey agar, M4 agar (selective for *Streptococcus* spp.), and Ektoen agar (selective for Enterobacteriaceae), and the plates were incubated for another 24 h. Colonies were identified using a classical procedure and appropriate API Sugar sets (Bio-Mérieux, Marcy l'Étoile, France) and Crystal (Becton Dickinson, Erembodegem-Aalst, Belgium). Isolation of at least one colony of one to three species of bacteria was considered to be an intramammary infection. If three or more bacterial species grew from any sample, that sample was considered contaminated and was excluded from the study. Because one negative QMS does not signify the absence of infection, and because *Sta. aureus* is shed in a cyclical manner from mammary glands (Sears et al., 1990), 2 or 3 consecutive samples were sometimes necessary for accurate diagnosis of infected quarters. As acute infections were frequently treated after the first sampling, only 1 sample was taken in acute mastitis-affected cows.

Total and Differential Cell Count

Immediately after milk collection, 50 ml of each sample was shipped to the Laboratory of the Milk Committee (Battice, Belgium), where a SCC was performed with a Fossomatic Automatic Cell Counter (Foss Electric, Hjøllerød, Denmark).

To determine the somatic cell type, high-SCC milk was diluted in PBS. Low-SCC milk was centrifuged (300 $\times g$) for 20 min at 4°C. Pellets were then suspended in 1 ml of PBS. Cell differentials were performed on 300- μ l cytopspin samples stained with May-Grundwald Giemsa (VWR, Leuven, Belgium). Neutrophils, lymphocytes, macrophages, and epithelial cells were differentiated according to their morphology. Three hundred cells were counted and the results of cell differentiation were expressed for each cell type as a proportion of the total number of cells counted.

Determination of Milk Eicosanoid Concentrations

Duplicate determinations of LXA₄ and LTB₄ milk concentrations were performed using specific ELISA (Neogen, Lexington, KY; range was 40 to 4000 pg/ml in the LTB₄ kit, 20 to 2000 pg/ml in the LXA₄ kit; cross-reactivities were <1 % for LTC₄ and LTD₄ and <0.1% for arachidonic acid in the LTB₄ kit, 1% for LXB₄ and <0.01% for LTB₄ in the LXA₄ kit). Radiolabeled LTB₄ (³H] LTB₄, Amersham Pharmacia Biotech, Gent, Belgium) was included to calculate recoveries which were always >90%. Acute QMS were fil-

tered with 70- μ m cell strainers (Becton Dickinson) in order to discard cell clusters. One milliliter of QMS was diluted with 1 ml of distilled water, and 1 ml of the mixture was loaded on a 100-mg C18 column (Varian, St-Katelijne-Waver, Belgium) after conditioning with 2 ml of distilled water followed by 2 ml of methanol. The column was subsequently washed with 1 ml of each of the following substances: distilled water, methanol:distilled water (30:70), and hexane. The column was centrifuged at 3200 $\times g$ for 3 min to withdraw any trace of hexane. Finally, eicosanoids were eluted from the C18 column with 1 ml of methanol. The collected eluate was evaporated to dryness under a stream of nitrogen. Dried samples were reconstituted in an appropriated volume of assay buffer.

Statistical Analysis

Data are presented as means $\pm$ standard errors of the mean. The differences between mean values were estimated using an ANOVA with subsequent Fisher's protected least significant difference test. Linear associations between variables were assessed by the use of standard least squares linear regressions. Correlation coefficients were presented as measures of linear association for regression relationships. Significant differences of the slopes from zero were determined using two-tailed Student's *t*-test. A *P*-value < 0.05 was considered significant.

RESULTS

Sample Characterization

Cellular characteristics of milk samples recovered from healthy, acute, and chronic mastitis-affected cows are provided in Table 1. The SCC in milk samples from healthy cows was always <10⁵ cells/ml. Conversely, mastitis was accompanied by a drastic increase in SCC, which was always >10⁷ in the acute form of the disease and ranged from 4.31 $\times$ 10⁵ to 10⁷ cells/ml in the chronic form. Differential cell counts showed a significant increase in the percentage of neutrophils associated with a significant decrease in the percentages of lymphocytes and macrophages in milk samples from cows suffering from mastitis compared with healthy cows.

Table 2 shows bacterial species isolated from milk samples of healthy and acute and chronic mastitis-affected quarters. Some quarters were infected by two organisms. Only one healthy quarter was shown to contain an infectious agent, namely *Sta. aureus*. *Streptococcus uberis* was the main pathogen isolated in acute infections (37.5%). Interestingly, no bacteria were found in 2 of the 8 acute quarters. Maybe using

Table 1. Cellular characteristics of milk recovered from healthy, chronic and acute mastitis-affected quarters (data are presented as mean $\pm$ SEM).

	Healthy (n = 25)	Chronic (n = 40)	Acute (n = 8)
SCC $\times$ 1000/ml	40.0 $\pm$ 4.5 [†]	2019.8 $\pm$ 350.1* [†]	>9999*
Macrophages, %	35.2 $\pm$ 4.7 [†]	19.3 $\pm$ 1.9*	10.0 $\pm$ 3.6*
Lymphocytes, %	18.4 $\pm$ 3.6 [†]	1.4 $\pm$ 0.8*	0.3 $\pm$ 0.3*
Neutrophils, %	34.2 $\pm$ 7.5 [†]	71.1 $\pm$ 2.8* [†]	88.5 $\pm$ 3.2*
Epithelial cells, %	12.2 $\pm$ 3.0 [†]	8.2 $\pm$ 1.1	1.2 $\pm$ 1.2*

*Significantly different from the values obtained with samples of healthy quarters ($P < 0.05$).[†]Significantly different from the values obtained with samples of acute quarters ($P < 0.05$).

a single milk sample, rather than three, reduced the sensitivity of diagnosis in this group. Pathogen bacteria were detected in all chronic mastitis-affected quarters, with *Str. uberis* being the main infectious agent present in those quarters (34%). In this form of the disease, the incidence of *Sta. aureus* was high, reaching 27.7%.

LXA₄ and LTB₄ Concentrations in Healthy and Mastitis-Affected Quarters

As expected, LTB₄ values were low in milk from healthy quarters and were drastically increased in the acute mastitis-affected quarters (Figure 1A). In chronic infections, LTB₄ concentrations reached intermediate levels, which were significantly different from those of healthy and acute mastitis-affected quarters (Figure 1A). The LXA₄ concentrations were always lower than the LTB₄ concentrations. As observed for LTB₄ concentrations, LXA₄ values were low in milk from healthy quarters and were highly increased in the acute mastitis-affected quarters (Figure 1B). However, in chronic mastitis-affected quarters, LXA₄ concentrations were comparable to those recorded in healthy quarters ($P = 0.95$), so that the LXA₄:LTB₄ ratio was significantly lower in chronic quarters compared with those observed in healthy and acute mastitis-affected quarters, which were not significantly different ($P = 0.37$) (Figure 1C). These results show the existence of an imbalance between LXA₄ and LTB₄ in the chronic inflammation of the bovine udder, which could be due to a defect of LXA₄ synthesis.

Relationship Between LXA₄-LTB₄ and SCC in the Chronic Mastitis-Affected Quarters

To better characterize chronic mastitis, in terms of LTB₄ and LXA₄ production, QMS recovered from chronically affected quarters were categorized into 5 groups according to the SCC measured at sampling time (group 1, $4 \times 10^5 \leq \text{SCC} < 7 \times 10^5$; group 2, $7 \times 10^5 \leq \text{SCC} < 10^6$; group 3, $10^6 \leq \text{SCC} < 2 \times 10^6$; group 4, $2 \times 10^6 \leq \text{SCC} < 4 \times 10^6$; group 5, $\text{SCC} \geq 4 \times 10^6$ cells/ml). The values obtained in the 5 groups were compared with those from healthy and acute mastitis-affected quarters. Cellular characteristics of samples recovered from the five groups of quarters are provided in Table 3. As expected, neutrophil percentages gradually increased with the number of somatic cells, whereas macrophage percentages decreased. Lymphocyte percentages remained low in all categories. Bacterial species isolated from samples obtained in the five groups are shown in Table 4. Some quarters were infected by two organisms.

Eicosanoid concentrations measured in the 5 categories of chronic mastitis-affected quarters were compared with each other and with the concentrations recorded in healthy and acute mastitis-affected quarters (Figure 2). The LTB₄ values from groups 1 to 4 were approximately twofold higher than those from healthy quarters (Figure 2A). Mean LTB₄ concentrations in group 5 were drastically higher than those of the other groups, but were significantly lower than the acute mastitis-affected quarters mean (Figure 2A). The means of LXA₄ concentrations recorded in

Table 2. Bacterial species isolated from milk samples of healthy, chronic and acute mastitis-affected quarters.

Bacterial species isolated	Healthy (n = 25)		Chronic (n = 40)		Acute (n = 8)	
	n	%	n	%	n	%
No bacterial growth	24	96.0	0	0	2	25.0
Coagulase-negative staphylococci	0	0	15	31.9	1	12.5
<i>Streptococcus uberis</i>	0	0	16	34.0	3	37.5
<i>Streptococcus agalactiae</i>	0	0	3	6.4	0	0
<i>Staphylococcus aureus</i>	1	4.0	13	27.7	0	0
<i>Escherichia coli</i>	0	0	0	0	2	25.0

Figure 1. Mean leukotriene (LT) B₄ (A) and lipoxin (LX) A₄ (B) concentrations and LXA₄:LTB₄ ratio (C) in milk obtained from healthy (n = 25), chronic (n = 40), and acute (n = 8) mastitis-affected quarters. Data are presented as means ± SD. *Significantly different from the mean obtained with samples of healthy quarters ($P < 0.001$). †Significantly different from the mean obtained with samples of acute quarters ($P < 0.001$).

Table 3. Cellular characteristics of milk recovered from chronic mastitis-affected quarters categorized according to the somatic cell count measured at sampling time.

	Group 1	Group 2	Group 3	Group 4	Group 5
SCC groups × 1000/ml	400–699 n = 9	700–999 n = 9	1000–1999 n = 10	2000–3999 n = 6	>4000 n = 6
SCC × 1000/ml	520.6 ± 25.8	822.4 ± 33.6	1425.7 ± 71.4*	2526.8 ± 132.3*†	6547.5 ± 981.9*†
Macrophages, %	20.1 ± 3.9	20.4 ± 2.8	17.6 ± 4.1	15.8 ± 3.1	14.8 ± 1.9
Lymphocytes, %	3.1 ± 2.5	0.4 ± 0.4	0.6 ± 0.3	0.5 ± 0.3	1.4 ± 0.6
Neutrophils, %	66.8 ± 7.2	68.2 ± 3.1	74.4 ± 3.8	77.8 ± 3.6	80.6 ± 2.6
Epithelial cells, %	10.0 ± 2.4	11.0 ± 3.0	7.4 ± 1.7	5.9 ± 4.5	3.2 ± 1.5*

Data are presented as mean ± SEM.

*Significantly different from the values obtained with samples of group 1 ($P < 0.05$).

†Significantly different from the preceding value ($P < 0.05$).

Table 4. Bacterial species isolated from milk of chronic mastitis-affected quarters categorized according to the somatic cell count measured at sampling time.

	Group 1	Group 2	Group 3	Group 4	Group 5
SCC groups × 1000/ml	400–699	700–999	1000–1999	2000–3999	>4000
Bacterial species isolated	n = 9	n = 9	n = 10	n = 6	n = 6
No bacterial growth					
n	0	0	0	0	0
%	0	0	0	0	0
Coagulase-negative Staphylococci					
n	4	5	3	2	1
%	44.4	41.7	27.3	28.6	12.5
<i>Streptococcus uberis</i>					
n	1	3	4	3	5
%	11.1	25.0	36.4	42.9	62.5
<i>Streptococcus agalactiae</i>					
n	1	1	0	1	0
%	11.1	8.3	0	14.3	0
<i>Staphylococcus aureus</i>					
n	3	3	4	1	2
%	33.3	25.0	36.4	14.3	25.0

Figure 2. Mean leukotriene (LT) B₄ (A) and lipoxin (LX) A₄ (B) concentrations and LXA₄:LTB₄ ratio (C) in milk obtained from chronic mastitis-affected quarters categorized according to the somatic cell count measured at sampling time. These values were compared to those obtained from healthy and acute mastitis-affected quarters (empty columns). 1) $4 \times 10^5 \leq \text{SCC} < 7 \times 10^5$, 2) $7 \times 10^5 \leq \text{SCC} < 10^6$, 3) $10^6 \leq \text{SCC} < 2 \times 10^6$, 4) $2 \times 10^6 \leq \text{SCC} < 4 \times 10^6$, 5) $\text{SCC} \geq 4 \times 10^6$ cells/ml. Data are presented as means $\pm$ SD. *Significantly different from the mean obtained with samples of healthy quarters ($P < 0.001$). †Significantly different from the mean obtained with samples of acute mastitis-affected quarters ($P < 0.001$). S = significantly different from $P < 0.01$.

the 5 chronic groups were all similar to the values obtained in the healthy quarters (Figure 2B). The LXA₄:LTB₄ ratio in each group of chronic mastitis-affected quarters was thus significantly lower than that of healthy quarters (Figure 2C). Only the LXA₄:LTB₄ ratio of group 1 was not significantly lower ($P = 0.35$) than that of the acute mastitis-affected quarters (Figure 2C). These results confirm that whatever the SCC, an imbalance between LXA₄ and LTB₄ concentrations occurs in the chronic inflammation of the bovine udder because chronic mastitis-affected cows display lower LXA₄ concentrations than acute mastitis-affected cows for these potentially protective lipid mediators.

Next, we studied the relationships between each eicosanoid and the degree of udder inflammation as assessed by SCC measurement in chronic mastitis-affected quarters. Accordingly, linear regressions were carried out to assess the relationship between each eicosanoid concentration and SCC, but also between each eicosanoid and the number of neutrophils and macrophages present in milk samples (Figure 3). These regression analyses showed that LTB₄ concentration measured in chronic mastitis-affected quarters was highly correlated to SCC, neutrophil, and macrophage numbers. A lower but significant correlation was also observed between LXA₄ concentrations and SCC, and neutrophil and macrophage numbers. For each eicosanoid, the highest correlation was observed when comparing LXA₄ and LTB₄ concentrations with neutrophil number ($r = 0.84$ for LTB₄ and $r = 0.53$ for LXA₄).

No significant correlation was found between eicosanoids concentrations in milk samples and age, stage of lactation, or pathogen.

DISCUSSION

Persistent accumulation of inflammatory cells in the udder, with neutrophil being the predominant cell type, is a characteristic feature of chronic mastitis in dairy cows. Leukotriene B₄ is a potent chemotactic agent, known to induce recruitment and accumulation of neutrophils in the bovine mammary gland. Leukotriene-stimulated neutrophil functional responses are opposed by LX which represent a unique class of lipid mediators with potent anti-inflammatory actions, like inhibition of neutrophil recruitment by attenuating their chemotaxis, adhesion, and transmigration across vascular and endothelial cells (Serhan, 1997). Moreover, it has been recently demonstrated that LXA₄, aspirin-triggered 15-epi-LXA₄, and their stable analogs were able to downregulate IL-8 secretion and subsequent recruitment of neutrophils by intestinal

epithelia (Gronert et al., 1998). This was due to down-regulation of proinflammatory gene expression via inhibition of the nuclear factor κ B (NF- κ B) pathway (Gewirtz et al., 2002). This observation is of particular interest because most of the genes encoding inflammatory proteins involved in neutrophil migration and activation have been shown to contain κ B sites for NF- κ B within their promoter, and therefore to partly depend on NF- κ B for their expression (Pahl, 1999). Boulanger et al. (2003) have recently demonstrated that NF- κ B activity was increased in milk cells from chronic mastitis-affected cows.

All of these considerations led us to hypothesize that the chronic inflammation of the udder could be associated with an unfavorable ratio between these two eicosanoids and that the persistence of neutrophil accumulation could be due to an increase in LTB₄ synthesis and/or an impaired LXA₄ production. In the present study, we have measured LXA₄ and LTB₄ concentrations and their ratio in milk of healthy, acute, and chronic mastitis-affected quarters. We showed that LTB₄ and LXA₄ concentrations were low in healthy quarters but were drastically increased in acute mastitis. In chronic mastitis-affected quarters, although LTB₄ concentrations increased with the SCC, LXA₄ concentrations remained at levels similar to those found in healthy quarters. Only a slight increase in LXA₄ concentrations was observed with the highest SCC. Regression analysis confirmed these observations, showing a high correlation between LTB₄ and SCC, and a lower, but significant, correlation between LXA₄ and SCC. We therefore found that LXA₄:LTB₄ ratio was significantly lower in chronic mastitis-affected quarters when compared with healthy and acute mastitis-affected cows. This imbalance could be implicated in the pathogenesis of the chronic bovine mastitis and might explain, at least in part, the persistent accumulation of neutrophils in milk from chronic mastitis-affected cows.

Formation of native LX has been shown in several *in vivo* studies, such as in nasal lavage fluids from aspirin-sensitive asthmatics (Levy et al., 1993), in experimental nephritis (Papayianni et al., 1995), or in crevicular fluids from localized juvenile periodontitis patients (Pouliot et al., 2000). Moreover, it has been demonstrated that asthmatic patients possess the capacity to generate both LXA₄ and 15-epi-LXA₄, but aspirin-intolerant asthmatics' whole blood showed reduced LX and 15-epi-LX biosynthetic capacity and lower ratios for LXA₄:LTC₄ generation when compared with values obtained for aspirin-tolerant asthmatics and healthy patients (Sanak et al., 2000). Here, we document that milk LXA₄ concentrations from chronic mastitis-affected cows are approximately sev-

Figure 3. Relationship between leukotriene (LT) B_4 and lipoxin (LX) A_4 concentrations and SCC (A) and neutrophil (B) and macrophage numbers (C) in milk samples obtained from chronic mastitis-affected quarters.

enfold lower than those from acute mastitis-affected cows, and are similar to those from healthy cows, suggesting that these diminished LXA_4 levels may play a role in the mechanisms by which inflammation be-

comes persistent. Several hypotheses that are not mutually exclusive might account for the low LXA_4 concentrations measured in chronic mastitis-affected cows. In humans, LX biosynthesis is an example of LO

interaction via transcellular routes (Serhan, 1994). Lipoxins can be generated by 1 of 3 routes that can be operative either independently or in concert, since these biosynthetic pathways are assembled during cell-cell interaction and/or when cells are primed by cytokines (Serhan, 1997). Thus, we may first assume that neutrophils or other cell types are not correctly primed in this model of chronic inflammation. Second, biosynthetic pathways generating LX might be deficient or inhibited. It has been demonstrated, for example, that platelets lose their ability to generate LX during chronic myelogenous leukemia (Stenke et al., 1991). So, the question of whether neutrophils or other cell types lose, partly, their ability to generate LXA₄ in chronic mastitis is addressed. Third, another explanation might result from inappropriate distribution within the tissues. It may be hypothesized that LXA₄ generated in blood from interaction with endothelial cells may not be able to diffuse within the alveolous lumen in the context of a chronic inflammation. Finally, we may also assume that an increase in the LX catabolism occurs in chronic mastitis, thus leading to local decrease in LXA₄ concentrations, but without a diminished biosynthetic capacity.

In view of the present findings, further studies are needed to clearly elucidate the mechanisms that explain why lower LXA₄ concentrations were found in the milk of chronic mastitis-affected cows and to determine whether administration of LX or stable analogs could have therapeutic potential in the treatment of chronic bovine mastitis.

CONCLUSIONS

The present study shows for the first time the existence of an LXA₄:LTB₄ imbalance in chronic mastitis-affected cows because of low LXA₄ concentrations. Since LXA₄ has many anti-inflammatory properties, our study supports the idea that these diminished LXA₄ levels, compared with acute mastitis, could be involved in chronic mastitis pathogenesis and that administration of LX or stable analogs could have therapeutic interests in readjustment of the inflammatory imbalance occurring in the chronic inflammation of the bovine mammary gland.

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