Reduction of Acyloxyacyl Hydrolase Activity in Circulating Neutrophils from Cows After Parturition

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ABSTRACT

Bovine neutrophils contain the enzyme acyloxyacyl hydrolase, which hydrolyzes the acyloxyacyl linkage of the two nonhydroxylated fatty acyl chains to two 3-hydroxy fatty acids in the highly conserved lipid A part of endotoxins with high specificity. This hydrolysis decreases the toxicity of lipid A, but the immunostimulatory capacity of endotoxins is largely maintained. In two trials, we studied the activity of acyloxyacyl hydrolase in neutrophils that had been isolated from the blood of 18 dairy cows around parturition. Between 10 and 26 d after parturition, the activity of acyloxyacyl hydrolase in neutrophils decreased approximately 20% below prepartum activity. At about 2 mo after parturition, acyloxyacyl hydrolase activity returned to prepartum values. Changes in acyloxyacyl hydrolase activity could not be attributed to changes in binding of lipopolysaccharides by the CD14 molecules on neutrophils or monocytes. We hypothesize that decreased acyloxyacyl hydrolase activity in neutrophils shortly after parturition is a factor that increases the susceptibility of dairy cows to coliform mastitis during early lactation.

(Key words: mastitis, neutrophils, endotoxin)

Abbreviation key: AOAH = acyloxyacyl hydrolase, LPS = lipopolysaccharides.

INTRODUCTION

Endotoxins are the lipopolysaccharides (LPS) from Gram-negative bacteria. These LPS are released upon bacterial lysis and bind first to LPS-binding proteins in the circulation. The LPS-binding protein complex then interacts with the CD14 molecule on monocytes, macrophages, and neutrophils (15, 30, 32). Priming of neutrophils by LPS is thought to be accomplished predominantly by an interaction that is dependent on CD14 (26). Lipopolysaccharide binds to CD14 on neutrophils in the presence of serum, and when no serum is present, other receptors are involved (14). Bovine neutrophilic CD18 may also be important as a receptor for LPS (25). After ingestion of LPS by neutrophils, the highly conserved lipid A moiety, which is associated with most of the toxic effects of endotoxins, is deacylated and dephosphorylated. Both reactions detoxify the molecule while leaving the antigenic sugar epitopes intact. The lipid A molecule is a glucosamine disaccharide that is substituted by four 3-hydroxy fatty acids, two of which are further substituted by nonhydroxylated acyl chains in acyloxyacyl linkage.

Acyloxyacyl hydrolase (AOAH) specifically hydrolyzes the two acyloxyacyl linkages (20). The general effectiveness of detoxification is suggested by the presence of endotoxins only in milk from cows with the most severe cases of coliform mastitis (1).

Many of the effects of mastitis caused by Escherichia coli can be mimicked by administration of E. coli endotoxin, suggesting that the endotoxin is important in the pathology of coliform mastitis (4, 36, 37). Consequently, endotoxin has been used experimentally to induce a sterile mastitis in dairy cows. However, the endotoxin-mastitis model is only partially comparable with E. coli mastitis. Because clinical symptoms differ between intravenous and intramammary injections of endotoxin, it has been suggested (12, 13) that endogenous mediators other than endotoxins are released and absorbed from the inflamed udder. Nonetheless, endotoxins are clearly important in the pathogenesis of acute coliform mastitis, either directly or indirectly.
During early lactation, a spectrum of detrimental responses to coliform infection of the mammary gland occurs among dairy cows (2). In contrast, during midlactation, intramammary injection of LPS causes an inflammatory reaction without detrimental effects (3, 12, 13, 25, 29). The objective of the present investigation was to determine whether deacylation of endotoxins by neutrophil AOAH is impaired during the period shortly after parturition.

**MATERIALS AND METHODS**

**Cows and Experimental Schedule**

Eighteen cows in two trials served as sources of blood neutrophils. The first trial used 7 Holstein cows from the USDA dairy herd (Beltsville, MD) in fifth (n = 4), third (n = 1), and second (n = 2) lactations. Their 305-d milk yield was 10,064 ± 375 kg. All cows were free of intramammary pathogens at the start of each trial. For diagnostic bacteriology (24), aseptically collected foremilk samples were obtained prior to parturition and on each day of postpartum blood collection. Additionally, SCC of foremilk samples were determined by electronic counting. One blood sample was taken approximately 1 wk before calving, three times per week during the first 4 wk postpartum, and once per week during postpartum wk 5 to 8. Neutrophils were immediately isolated from these blood samples.

**Isolation of Neutrophils**

All solutions used in this study were determined to be free of pyrogens using a limulus amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD). Neutrophils were isolated for AOAH determinations as follows. Forty milliliters of blood from the coccygeal vein were collected into vacutainer tubes containing an equal volume of Alsever’s solution (Gibco BRL, Life Technologies, Grand Island, NY) as anticoagulant. The diluted blood was transferred to 50-ml plastic centrifuge tubes and centrifuged for 10 min at 300 g. The supernatant was decanted and the cells were resuspended in 10 ml of 0.01 M PBS, the erythrocytes were lysed again, as just described. The remaining leukocytes were centrifuged at 600 × g for 5 min at 4°C and then were washed with PBS.

Neutrophils were isolated for flow cytometric analysis of the CD14 molecule as follows. Fourteen milliliters of blood from the coccygeal vein were collected into vacutainer tubes containing an equal volume of Alsever’s solution (Gibco BRL, Life Technologies, Grand Island, NY) as anticoagulant. The diluted blood was transferred to 50-ml plastic centrifuge tubes and centrifuged for 10 min at 300 × g. Erythrocytes in the pellet were lysed by resuspension in 20 ml of an ice-cold sterile buffered solution (21 mM Tris and 0.14 M NaCl) and incubation for 10 min at 4°C. After centrifugation (200 × g for 10 min), the cells were washed thrice with Alsever’s solution. Final pellets were resuspended in RPMI 1640 (Gibco BRL), containing 1% BSA and 0.2% NaN₃, at a final concentration of 10⁷ cells/ml.

**Determination of AOAH Activity**

The number of neutrophils in suspension was determined with an electronic cell counter (Multisizer II; Coulter Electronics Inc., Hialeah, FL); viability was estimated by trypan blue exclusion, and the neutrophil percentage was determined microscopically using Wright-stained smears. Typically, 95% of the cells were neutrophils, and viability was 98%. To 10⁶ neutrophils, 100 μl of lysis buffer [100 mM KCl, 3.9 mM NaCl, 3.5 mM MgCl₂, 15 mM Na₂EDTA, 10 mM HEPES, 1% (vol/vol) Nonidet P-40; (Sigma Chemical Co., St. Louis, MO) and 75 μg of phenylmethylsulfonylfluoride/ml; pH 7.4]) were added. After incubation at room temperature for 10 min, the suspension was centrifuged for 10 min at 2000 × g, and the supernatant containing the AOAH was stored at -80°C until assayed.

Salmonella typhimurium PR122 was radiolabeled with [³H]acetate and N-acetyl-[¹⁴C]glucosamine (New England Nuclear Corp., Boston, MA) as previously described (7), and radiolabeled LPS was extracted by the method of Galanos et al. (6). The LPS substrate was suspended in 0.1% triethylamine at a concentration of 1 mg/ml and stored at -80°C. One milligram of LPS substrate contained 19,421 dpm of [³H] and 2356 dpm of [¹⁴C]. Before determination of AOAH activity, the LPS substrate was thawed and suspended at a concentration of 2.5 μg/ml in a reaction mix, pH 5.5, that contained 150 mM NaCl, 5 mM
CaCl₂, 0.1% Triton X-100, 20 mM Tris-citrate, and 1 mg of BSA/ml. Fifty microliters of the neutrophil lysates containing AOAH and 50 µl of PBS were added to 400 µl of reaction mix containing substrate (1 µg per tube) and incubated for 15 h at 37°C. After incubation, fatty acids were extracted with a chloroform and methanol mixture as described by Munford and Erwin (21), except that radioactivity in an aliquot of a single extraction was quantified. Preliminary data had indicated that results of a single extraction were within 2% of those obtained by double extraction. An aliquot of the chloroform phase was evaporated to dryness under a stream of air. Then, 0.2 ml of an aqueous solution of SDS and EDTA (3.5 mM SDS and 1 mM Na₂EDTA) and 5 ml of scintillation fluid were added for quantification of radioactivity.

Radioactivity was quantified by liquid scintillation counting, using protocols for quench correction of dual-labeled samples. Because 14C is present only in the LPS backbone, 14C serves as a measure of contamination of the free 3H-labeled fatty acid extract with unhydrolyzed LPS. Consequently, the 3H content of the fatty acid fraction was corrected for the presence of small quantities of 14C as follows: 3H disintegrations per minute corrected = 3H disintegrations per minute measured - (14C disintegrations per minute × 3H disintegrations per minute/14C disintegrations per minute in the LPS). The ratio of 3H to 14C disintegrations per minute in the LPS substrate was 8.24. The mean value for specific activity of the fatty acids in the LPS substrate was 26.4 dpm/nmol, and 1 ml of enzyme suspension was derived from 10⁷ neutrophils. Thus, the picomoles of fatty acids in the LPS substrate was 8.24. The mean value for specific activity of the fatty acids in the LPS substrate was 26.4 dpm/nmol, and 1 ml of enzyme suspension was derived from 10⁷ neutrophils. Thus, the picomoles of fatty acids released by AOAH per hour per 10⁷ neutrophils were 3H disintegrations per minute corrected × (1 nmol/26.4 dpm) × (1000 pmol/nmol) × (1/15 h) × (1/0.05 ml). Interassay and intraassay coefficients of variation were below 4%.

Immunostaining and Flow Cytometry

Aliquots of cell suspension (100 µl; 1 × 10⁶ cells) were transferred to 75-mm × 12-mm polystyrene test tubes and were concentrated by centrifugation (200 × g for 10 min at 4°C). Cells were incubated with 100 µl of RPMI 1640 (control) or 100 µl of bovine anti-CD14 (BioSource International, Camarillo, CA; dilution 1:20 with RPMI 1640) for 30 min on ice and in the dark. Cells were then centrifuged (200 × g for 10 min at 4°C) and washed twice in RPMI 1640 by centrifugation (200 × g for 10 min at 4°C). Cells were incubated on ice in the dark for 30 min with 100 µl of second antibody (goat anti-mouse IgG-fluorescein isothiocyanate-conjugate; Sigma Chemical Co.; dilution 1:500, vol/vol) with RPMI 1640. The cells were collected and washed twice by centrifugation (200 × g for 10 min at 4°C) in PBS. Finally, the cell pellet was fixed in 2 ml of 1% paraformaldehyde in PBS (pH 7.5) and was stored at 4°C in the dark until flow cytometry.

Flow cytometric analysis was performed using an EPICS 741 flow cytometer (Coulter Electronics Ltd., Luton, United Kingdom) equipped with a 5-W argon ion laser (Coherent, Palo Alto, CA). ISOTON II (Coulter Electronics Ltd.) was used as the sheath fluid.

Analyses were performed on 15,000 cells with the laser operating at 250 mW at 488 nm. Fluorescence emission was captured with a 525-nm band pass filter. Individual cells were classified according to cell type based upon forward light scatter, which is related to cell size, and orthogonal light scatter, which is related to cell granularity. Dot plots were gated for monocytes and polymorphonuclear leukocytes. The expression of the CD14 molecule (binding of CD14 antibody) was measured as the percentage of cells that fluoresced and the logarithmic mean fluorescent channel, a measure of fluorescence intensity.

Statistical Analysis

Duplicate determinations of enzyme activity were averaged and analyzed by PROC MIXED of SAS® (28). Day of sample relative to parturition was grouped into nine time periods: prepartum and postpartum d 1 to 3, 5 to 7, 10 to 12, 14 to 17, 19 to 21, 24 to 26, 28 to 36, and 42 to 56. The model used for analysis of AOAH activity included as main effects cow, period, trial, and the interaction between period and trial. Flow cytometry data were similarly analyzed. However, because these data were generated from a single trial, the model included only cow and period as main effects.

RESULTS AND DISCUSSION

The activity of AOAH in circulating neutrophils prior to parturition averaged 122 pmol of fatty acids/10⁷ neutrophils per hour (Figure 1). After parturition, AOAH activity decreased (P < 0.01) to 100, 98, and 100 pmol of fatty acids/10⁷ neutrophils per hour during postpartum periods d 10 to 12, 14 to 17, 19 to 21, and 24 to 26, respectively. The AOAH activity returned to 119 pmol of fatty acids/10⁷ neutrophils per hour by 42 to 56 d postpartum, when
AOAH activity did not differ from activity prior to parturition (P > 0.1).

The effect of period on AOAH activity was not due to artifacts of neutrophil isolation or to infection status. Because both neutrophils (7) and monocytes (22) have AOAH activity, the effectiveness of the procedure for neutrophil isolation could be a source of variability. However, in the neutrophil preparations used in the present study, neutrophil purity ranged between 85 and 98%, there were few or no monocytes, and there was no effect of period (P > 0.1). Thus, the observed effects must be attributed to changes in neutrophil AOAH activity rather than to contamination of neutrophil preparations with varying quantities of monocytes or other leukocytes. Additionally, during bacterial infection of the mammary gland, AOAH activity in milk neutrophils is increased (19).

In our experiment, cows occasionally had minor bacterial infections from coagulase-negative staphylococci but never developed clinical symptoms. In the Beltsville trial, 1 cow had slightly elevated SCC (71,000 cells/ml) on d 5 postpartum, but never developed clinical mastitis, and had SCC <29,000 cells/ml on all other days. In the Ghent trial, there was no evidence of mastitis, and SCC never exceeded 50,000 cells/ml. Furthermore, because AOAH activity was measured in blood neutrophils, the changes in neutrophil AOAH activity that were observed in the present experiment were not likely due to inflammation within the udder.

Our results indicated that neutrophil AOAH activity in dairy cows was decreased during the early postpartum period. Although some variability in neutrophil AOAH activity among cows occurred shortly after parturition, the mean AOAH activity was consistently decreased approximately 20%, relative to prepartum values, during postpartum d 10 to 26 (P < 0.05). Neither the prepartum nor postpartum AOAH activities were correlated with milk yield (P > 0.05). The variability in AOAH activity among cows in this study was consistent with the variability in biological responses of cows during the early postpartum period to experimental infection of the udder with E. coli. The severe response of some cows to experimental coliform mastitis shortly after calving has been associated with impairment of neutrophil functions, such as chemotaxis (9, 11), diapedesis (34), respiratory burst (8, 11), and neutrophil counts in the blood (35). Impaired inactivation of endotoxin by neutrophils conceivably might contribute to a greater inflammatory response of the mammary gland to E. coli shortly after calving. Increased endotoxin concentrations might decrease neutrophil viability and contribute to the development of severe coliform mastitis.

In the defensive arsenal of neutrophils against LPS, AOAH is only one weapon. Lactoferrin is a glycoprotein that binds LPS and is synthesized and secreted by neutrophils (5). The concentration of lactoferrin in mastitic milk is six times higher than that occurring in normal milk (27). Other endotoxin-binding proteins synthesized by neutrophils are a protein that increases bactericidal and permeability activities (16), lysozyme (31), several cationic antimicrobial proteins (33), and the defensins (10). However, these proteins do not degrade the LPS molecule.

During experimental coliform mastitis, AOAH activity in milk neutrophils and in cell-free milk was strongly increased, and this increase was not due to de novo protein synthesis (18). This result stresses the importance of optimal AOAH activity in circulating neutrophils, which migrate rapidly into the milk upon infection of the mammary gland. Although secreted AOAH potentially affects the inactivation of LPS in the mammary gland, the optimum acidic pH of bovine AOAH (17) implies that the activity in milk would be lower than that in neutrophil granules and possibly negligible. Moreover, an inhibitor of AOAH has been detected in bovine milk (19). Intramammary injection of LPS results in an important decrease in the percentage of milk neutrophils expressing CD14, and CD18 expression is increased (25). The relative avidity of both receptors for LPS in milk and the capability of the receptors to enhance expression of AOAH have not been investigated.
TABLE 1. Expression of CD14 on neutrophils and monocytes.

<table>
<thead>
<tr>
<th>Period</th>
<th>Neutrophils</th>
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<th>Monocytes</th>
<th></th>
<th>Neutrophils</th>
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<th>Monocytes</th>
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<tr>
<td></td>
<td>Percentage of neutrophils and monocytes binding anti-CD14 monoclonal antibody.</td>
<td></td>
<td>Log mean fluorescence channel for anti-CD14 binding after nonspecific fluorescence of negative controls has been subtracted. The LMFC of negative controls (no primary antibody) was &lt;2.</td>
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<td>Prepartum</td>
<td>4 1 77 2</td>
<td>5.7 0.6 9.6 0.6</td>
<td>7.4 0.8 10.9 0.7</td>
<td>6.9 0.9 9.7 0.7</td>
<td>7.6 0.9 9.3 0.8</td>
<td>4.5 0.8 10.4 0.8</td>
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<tr>
<td>Postpartum</td>
<td>6 1 84 2</td>
<td>5.0 1.1 7.9 0.9</td>
<td>6.5 1.2 8.9 1.1</td>
<td>5.5 1.1 7.1 1.2</td>
<td>6.3 1.4 8.3 1.3</td>
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<td>1-3 d</td>
<td>6 1 79 3</td>
<td>5.0 1.1 7.9 0.9</td>
<td>6.5 1.2 8.9 1.1</td>
<td>5.5 1.1 7.1 1.2</td>
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<td>5-7 d</td>
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<td>14-17 d</td>
<td>3 1 79 3</td>
<td>5.0 1.1 7.9 0.9</td>
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<tr>
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<td>24-26 d</td>
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1Percentage of neutrophils and monocytes binding anti-CD14 monoclonal antibody.
2Log mean fluorescence channel for anti-CD14 binding after nonspecific fluorescence of negative controls has been subtracted. The LMFC of negative controls (no primary antibody) was <2.

The expression of the CD14 molecule on monocytes and neutrophils was examined during the peripartum period (Table 1). From 2 wk prior to parturition to 9 wk after parturition, no alterations (P > 0.1) were observed in the percentage of CD14-positive monocytes (80%) or neutrophils (5%), nor was the number of CD14 molecules (mean fluorescence) per monocyte or neutrophil altered (P > 0.1). Thus, changes in AOAH activity could not be attributed to changes in binding of LPS by the CD14 molecules on neutrophils or monocytes.

Around parturition, immature neutrophils appear in the blood. It is noteworthy that these neutrophils were observed in the blood of Holstein cows from 7 to 20 d postpartum (2), a period that approximately corresponds with the interval of decreased AOAH activity. However, because AOAH activity did not change when the percentage of immature neutrophils was increased following injection of bovine recombinant granulocyte colony-stimulating factor or LPS (18), alterations in neutrophil maturity do not appear to be responsible for changes in AOAH activity such as those observed in the present study. Furthermore, in the current study, neutrophil preparations contained a very small percentage of immature neutrophils, and there was no correlation between neutrophil maturity and AOAH activity (data not shown).

In this study, we demonstrated that deacylation of endotoxins by blood neutrophils is impaired shortly after parturition. We hypothesize that the decreased LPS deacylating activity of bovine neutrophils during early lactation may predispose dairy cows to severe coliform infections of the mammary gland during this period. Subjects for further investigation include the relationship between neutrophil AOAH activity and the severity of coliform mastitis shortly after calving and phagocytosis of LPS by bovine neutrophils.

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