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Intracellular and Extracellular Enzymatic Deacylation of Bacterial Endotoxin during Localized Inflammation Induced by *Escherichia coli*

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Acyloxyacyl hydrolase (AOAH), an enzyme that removes the secondary acyl chains of gram-negative bacterial lipid A (endotoxin), has been identified previously in human neutrophils and mouse macrophages. We report here that bovine leukocytes also contain AOAH activity. Although bovine AOAH deacylates bacterial lipopolysaccharide in a manner similar to human AOAH, it is active in vitro over a broader pH range, from 4.0 to 7.0. By using *Escherichia coli* infection of the bovine mammary gland as a model of localized gram-negative bacterial disease and associated tissue inflammation, AOAH activity per leukocyte increased. In addition, AOAH activity increased in the cell-free portion of infected mammary secretions. These data indicate that AOAH activity increases in leukocytes associated with inflammation induced by gram-negative bacteria and provide additional evidence of its potential involvement in the defense against the effects of bacterial endotoxin.

The lipid A component of bacterial endotoxin is believed to elicit many of the inflammatory events associated with gram-negative bacterial infection (11). Although antibiotics can combat gram-negative bacteria, successful therapy to detoxify lipid A has not been developed. Indeed, little is known about the catabolism of lipid A in vivo. Acyloxyacyl hydrolase (AOAH), a product of both human neutrophils (9) and mouse macrophages (12), deacylates lipid A by removing the nonhydroxylated fatty acyl chains (9), thus rendering the molecule less toxic (13). When tested in the dermal Shwartzman reaction, deacylated lipopolysaccharide (LPS) is at least 100-fold less potent than native LPS (13). In contrast, LPS enzymatically deacylated by AOAH retains much of the immunostimulatory potency of native LPS, as measured by a reduction by a factor of only 12 in the ability of deacylated LPS to stimulate murine splenocyte division compared with native LPS (13).

Bovine mastitis is an excellent model for studying localized bacterial infection because induction, collection, and isolation of inflammatory cells is relatively easy in this species. The bovine mammary glands function as separate, noncommunicating structures, facilitating the study of localized infection. Each mammary gland can serve as an experimental unit, with one gland being infected and another mammary gland from the same animal serving as a noninfected control (1). Furthermore, coliform mastitis is an important disease in dairy cattle, and a better understanding of its pathogenesis may improve prevention and treatment.

We report here the partial enzymatic deacylation of LPS by bovine leukocytes, similar to that seen in human neutrophils and murine macrophages. We measured bovine leukocyte AOAH activity over the course of acute gram-negative mastitis and show that activity per leukocyte increases. In addition, cell-free milk from infected mammary glands contains AOAH activity. These findings provide additional evidence that AOAH may play a role in modulating the effects of endotoxin in vivo.

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MATERIALS AND METHODS

LPS preparation. *Salmonella typhimurium* PR122 cells (deficient in glucosamine deaminase and UDP–glucose-4-epimerase [15]) were biosynthetically labeled with [2-^{3}H] acetate and *N*-acetyl-[1-^{14}C]glucosamine (New England Nuclear Corp., Boston, Mass.) in proteose peptone-beef extract broth as described previously (9). This *Salmonella* mutant incorporates GlcNAc into the rough-core region and lipid A regions of LPS. ^{3}H radiolabel is almost entirely located in the fatty acyl chains. Rough (Rc chemotype) LPS was prepared by growing strain PR122 in the absence of D-galactose and extracting the LPS by the method of Galanos et al. (8). The LPS was extracted several times with diethyl ether, suspended at a concentration of 1 μg/μl in distilled water that contained 0.1% triethylamine, and stored at −70°C. One microgram of LPS contained 35,000 dpm of ^{3}H-fatty acid and 8,300 dpm of [^{14}C]glucosamine (9). The average specific activity of the ^{3}H-fatty acids was 51,800 dpm/nmol, as determined by high-pressure chromatographic comparisons to known concentrations of fatty acid standards (see Fig. 3).

Induction of mastitis. An *Escherichia coli* strain isolated from a severe clinical case of bovine mastitis (J. Cullor) was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) for 16 h at 37°C. This strain, when inoculated into the mammary gland, produces a severe acute, reproducible mastitis that does not result in death of the animal. Bacteria were centrifuged at 2,000 × *g* for 20 min, washed with 10 mM sodium diphosphate buffer (pH 7.5), and diluted to 2 × 10^5 cells per ml. The left front mammary gland of each of seven adult Holstein cows was inoculated with 1.0 ml of this bacterial inoculum immediately following a milking. Inoculations were into the teat cistern via the teat canal with a teat canula attached to a 1.0-ml tuberculin syringe. The left rear gland served as a noninfected control. Mammary secretions were collected from infected and control glands at the time

of infection and at 4, 12, 24, 48, and 72 h postinfection. Heparinized blood samples were obtained at the same times by venipuncture of the jugular vein. Animals were given systematic antibiotics 72 h postinfection.

Bovine leukocyte (neutrophil) isolation. Peripheral blood neutrophils were isolated from heparinized bovine blood by selective centrifugation. In brief, 50 ml of whole blood collected in acid-citrate-glucose was centrifuged at 1,000 × *g* for 15 min, and the plasma and buffy coat were discarded. The remaining erythrocytes and neutrophils were washed with distilled water to lyse the erythrocytes, and isotonicity was then immediately restored by adding 2× phosphate-buffered saline (PBS; pH 6.8). The suspension was recentrifuged at 400 × *g* to pellet intact cells, the supernatant was removed, and 10 ml of distilled water was added to resuspend the pellet. Following gentle mixing for 30 s, isotonicity was again restored. The procedure was repeated twice to assure lysis of all erythrocytes.

The total number of cells isolated was determined by using a semiautomated cell counter (Coulter ZM; Coulter Inc., Hialeah, Fla.), and the percentage of neutrophils was determined by direct examination. The isolation technique routinely provided large numbers of neutrophils that were determined to be viable by trypan blue exclusion and greater than 95% neutrophil in content. The isolated leukocytes were lysed with a buffer developed by R. Munford, Southwestern Medical School (100 mM KCl; 3.9 mM NaCl; 3.5 mM $MgCl_2$; 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4] that contained 1% [vol/vol] Nonidet P-40, 15 mM EDTA, and 75 μg of phenylmethylsulfonyl fluoride per ml). The lysis buffer was added (0.1 ml/10^6 cells), and the cells were incubated at room temperature for 10 min with frequent mixing; then the mixtures were centrifuged at 2,000 × *g* for 10 min. Supernatant aliquots were stored at −70°C.

Mammary secretions were collected from individual glands into sterile containers. The number of cells per milliliter was determined by using an automated cell counter (Fossomatic; Foss Electric Corp., Hillerod, Denmark). After remaining at 4°C for no more than 1 h, the milk was centrifuged at 2,000 × *g* for 20 min, cream was removed from the cell-free supernatants, and the pelleted cells were washed with PBS (pH 7.4) and repelleted. The cells were then lysed and stored by using techniques described above for peripheral blood neutrophils. Aliquots of cell-free skim milk were also stored at −70°C. Cytologic examination of sedimented cells from noninfected and infected milk revealed that 66 and 99%, respectively, were neutrophils. The remaining cells consisted of lymphocytes, monocytes-macrophages, and epithelial cells in a ratio of 2:1.3:1.

AOAH activity quantification. AOAH activity in plasma, cell-free milk, and leukocyte lysates (peripheral blood and mammary) was measured by lipid extraction techniques previously described (9, 12). Activity was measured by the release of ^3H-labeled fatty acids (but not ^{14}C-labeled glucosamine) from double-radiolabeled LPS after incubation with enzyme for 16 h in a detergent-buffer solution (reaction mix). The reaction mix contained 0.1% Triton X-100, 20 mM Tris-citrate, 1 mg of bovine serum albumin per ml, 5 mM $CaCl_2$, and 150 mM NaCl. Reagents were prepared in pyrogen-free water and adjusted to pH 5.5. Radiolabeled LPS was added to the reaction mix (2.0 μl/ml) prior to each assay. Following incubation, fatty acids were extracted into chloroform-methanol (9). Enzyme activity in cell-free milk and in plasma was expressed as picomoles of fatty acid removed per milliliter of sample per hour. Enzyme activity in leukocyte lysates was expressed as pmol of fatty acid removed per 10^7 cells per h.

Human leukocyte lysates with AOAH activity were kindly provided by R. Munford.

Radioactivity measurements. Scintillation counting was performed by using a model Minaxi Tri-Carb 4000 Series counter (Packard Instrument Co. Inc., Laguna Hills, Calif.). Determination of ^3H radioactivity in samples included corrections for background events, as well as channel spillover (typically, 39% spillover from the ^{14}C to ^3H channels); quenching was determined by using an external standard and was corrected internally. The amount of ^3H-fatty acid released from cell-associated LPS was corrected for the presence of small amounts of acylated LPS in the chloroform extracts.

Thin-layer chromatographic (TLC) procedures. After incubation of double-radiolabeled LPS with cell-free infected milk for 16 h at 37°C, the released fatty acids were extracted into chloroform as previously described (9). In some experiments, the LPS was acid hydrolyzed prior to extraction as previously described (9). Aliquots were chromatographed on silica gel G plates with petroleum ether-diethyl ether-acetic acid (70:30:1) as the solvent. The plates were then sprayed with En^{3}Hance (New England Nuclear) and exposed to Kodak SB-5 film at −70°C.

HPLC procedures. For identification and quantification of individual fatty acids, phenacyl ester derivatives of the LPS were prepared (5). The derivatization products were separated by techniques previously described (6). Fatty acids were identified and quantitated by comparing retention times and peak areas with those of fatty acid standards. Fractions were collected from the high-performance liquid chromatographic (HPLC) column, and their ^3H contents were determined as described above.

Statistical analysis. Data were analyzed by univariant analysis of variance and Fisher's Least Significant Difference Test (16).

RESULTS

Demonstration of AOAH activity in bovine leukocytes and in infected milk. Bovine AOAH from peripheral blood leukocytes and cell-free milk, although identical in its enzymatic action to human AOAH, was active over a broader range of pHs. Incubation of preparations of radiolabeled LPS with bovine leukocyte lysates and cell-free infected bovine milk at various pH values showed that bovine AOAH was active from pH 4.0 to approximately pH 7.0, while human AOAH operates optimally in a narrower range (pH 4.5 to 5.5) (Fig. 1).

Bovine cell-free infected milk was incubated in a detergent-buffer solution with radiolabeled LPS substrate. The products of enzymatic deacylation of LPS were compared with fatty acids released by hydrolysis of LPS, by using TLC and HLPC. Bovine cell-free infected milk removes nonhydroxylated fatty acids from LPS, leaving the β-hydroxylated fatty acids attached to the glucosamine backbone (Fig. 2 and 3). When milk was heated to 80°C for 10 min and then incubated with LPS by using the same reaction conditions, deacylation of LPS did not occur.

The addition of increasing amounts of cell-free infected milk to a constant concentration of radiolabeled LPS resulted in a maximum deacylation of 26% (Fig. 4). Nonhydroxylated fatty acids comprised approximately 25% of the total fatty acids of this LPS preparation. The evidence was consistent with the presence of AOAH activity.

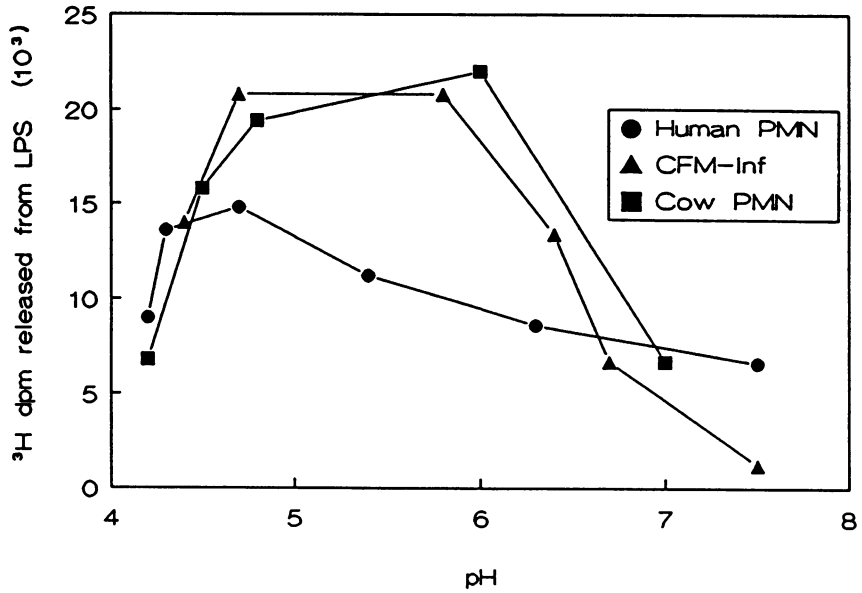

FIG. 1. Comparison of pH optimum curves for human and bovine AOAH. ^{3}H released from radiolabeled LPS after incubation with AOAH from human peripheral blood neutrophil lysate (Human PMN), bovine peripheral blood neutrophil lysate (Cow PMN), or bovine cell-free infected milk (CFM-Inf) was measured at various pH values.

AOAH activity in the mammary gland. All animals developed clinical mastitis; signs of systemic illness were noticeable at approximately 12 h postinfection. There was an influx of neutrophils into milk at this time (Fig. 5). Neutrophils accounted for 99% of the mammary leukocytes isolated from infected glands and 75 to 80% of leukocytes from noninfected glands. Signs of systemic illness were minimal by 24 h postinfection, but clinical mastitis was still observable at 72 h. Pure cultures of *E. coli* were reisolated from infected glands throughout the experimental period; noninfected glands remained sterile in all cases. Noninfected glands were observed to become inflamed (warmer than the noninfected glands, red, swollen, and indurated), with influx of neutrophils at 24 h postinfection (less than in infected glands).

AOAH activity remained low or undetectable in milk leukocyte lysates until 12 h postinfection in both infected and control (noninfected) glands. At this time, leukocyte AOAH activity began to rise in the infected glands, whereas the activity in leukocytes from control glands remained low (Fig. 6). AOAH activity in milk leukocytes from infected glands continued to rise throughout the 72-h experimental period. Milk leukocyte AOAH activity from infected glands was significantly greater ($P < 0.05$) than that found in leukocytes from noninfected glands for all times sampled after the initial infection. AOAH activity was increased slightly in the leukocytes from noninfected glands at 72 h postinfection. This increase was associated with the inflammatory reaction observed in the noninfected glands beginning at approximately 24 h postinfection.

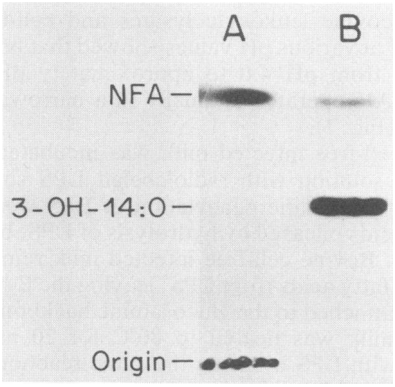

FIG. 2. TLC analysis of chloroform-soluble reaction products from hydrolyzed LPS and from enzymatically deacylated *S. typhimurium* LPS. Lane B, Fatty acids released from LPS by chemical hydrolysis; lane A, fatty acids released from LPS by bovine AOAH from cell-free infected milk. Counts located at the origin represent radiolabeled LPS that has carried over into chloroform. NFA, Nonhydroxylated fatty acids; 3-OH-14:O, 3-hydroxy-myristate.

Cell-free milk was also assayed for AOAH activity during mammary infection, and AOAH activity was easily detectable in infected glands (Fig. 5). There was a temporal correlation between the influx of leukocytes into the mammary gland during infection and the appearance of AOAH activity in cell-free milk. Cell-free milk from noninfected glands did not show significant AOAH activity until 72 h postinfection, when the leukocyte population in the milk had increased.

AOAH activity in peripheral blood. Differential cell counts in peripheral blood did not change similarly over the course of infection for all experimental animals (data not included). In general, animals were transiently neutropenic but did not show profound shifts toward neutrophil immaturity (left shift). AOAH activity increased slightly in peripheral blood leukocytes, beginning at approximately 48 h postinfection (Fig. 7). This increase was less than the change seen in AOAH activity in milk leukocytes. Analysis of plasma for AOAH activity failed to detect AOAH activity at any time.

DISCUSSION

The bovine mammary gland has proven to be a suitable model for the study of localized gram-negative infection and

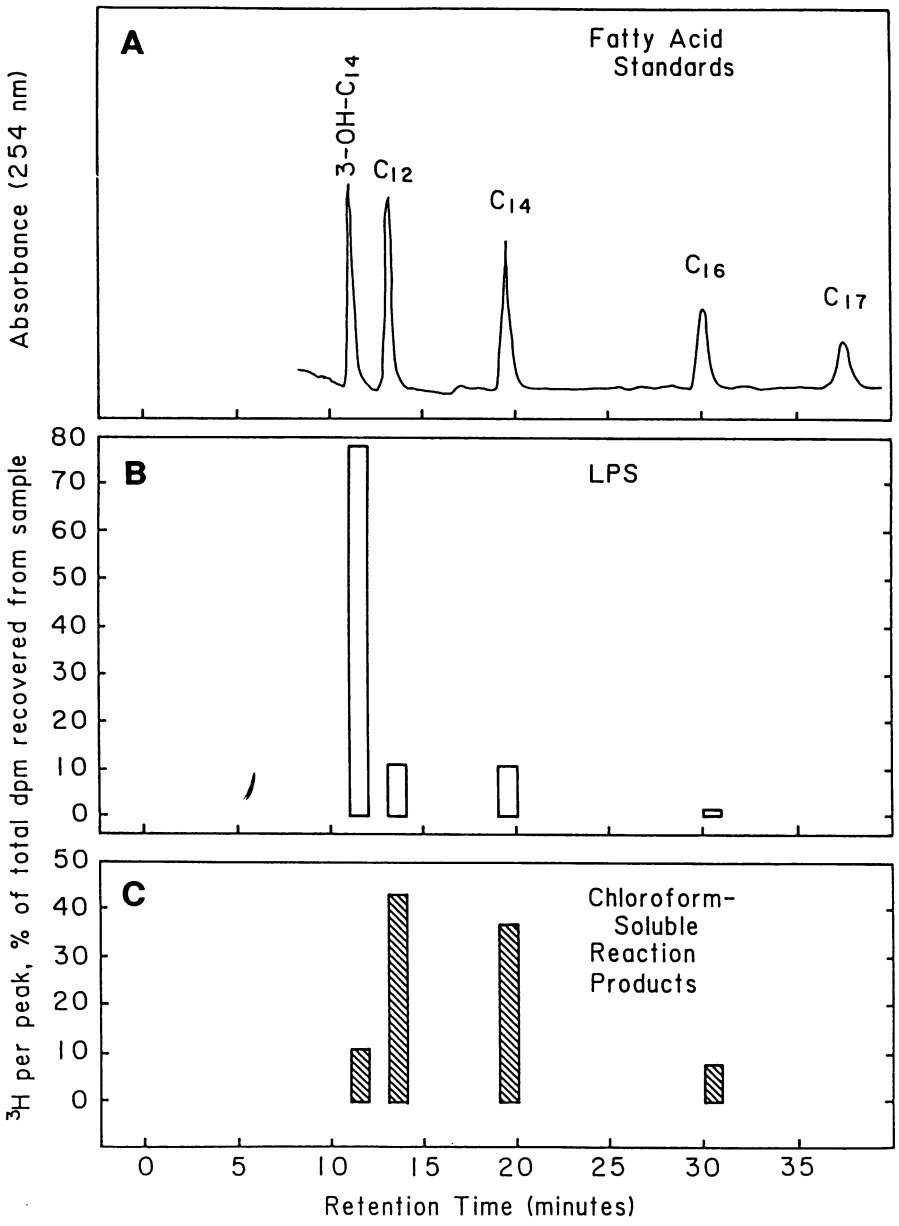

FIG. 3. HPLC separation of fatty acids from hydrolyzed LPS and from enzymatically deacylated LPS. (A) Peak profile of fatty acid standards. (B) Separation of chemically hydrolyzed LPS. (C) Effect of bovine AOAH on LPS. Bars represent counts collected from sequential fractions during HPLC separation.

associated changes in AOAH leukocyte activity. Each mammary gland functions as an independent secreting unit, and infection of one gland will not cause contamination of the other three noninfected glands (1). The bovine mammary gland is also a readily accessible site for the induction of gram-negative infection, as well as for the collection of large numbers of neutrophils following infection.

Bovine leukocytes expressed AOAH activity and deacylated LPS similar to human neutrophils. Although a human enzyme with AOAH activity has been purified (14), it is not clear if one or more enzymes are responsible for the AOAH activity in either human or bovine leukocytes. Both TLC and HPLC analyses showed that lysates of bovine blood neutrophils acted to remove nonhydroxylated fatty acids from the lipid A region of LPS while leaving the β-hydroxylated fatty acids attached (Fig. 2 through 4). However, bovine AOAH was active over a wider pH range than is the human enzyme. Human AOAH appears to retain activity when the pH is greater than 7, whereas bovine AOAH activity is less active at these pHs (Fig. 1). These differences in AOAH activity at various pH values may affect the animal's ability to deacylate LPS and may contribute to species differences in susceptibility to endotoxin.

Leukocyte AOAH activity increased during localized gram-negative infection. This increase can be attributed to an increase in enzyme activity per leukocyte isolated from the inflammatory focus, rather than solely to an increase in the number of leukocytes attracted to the site of infection (Fig. 6). AOAH activity has been detected in neutrophils (9) and in monocytes (12), and because we are unable to isolate

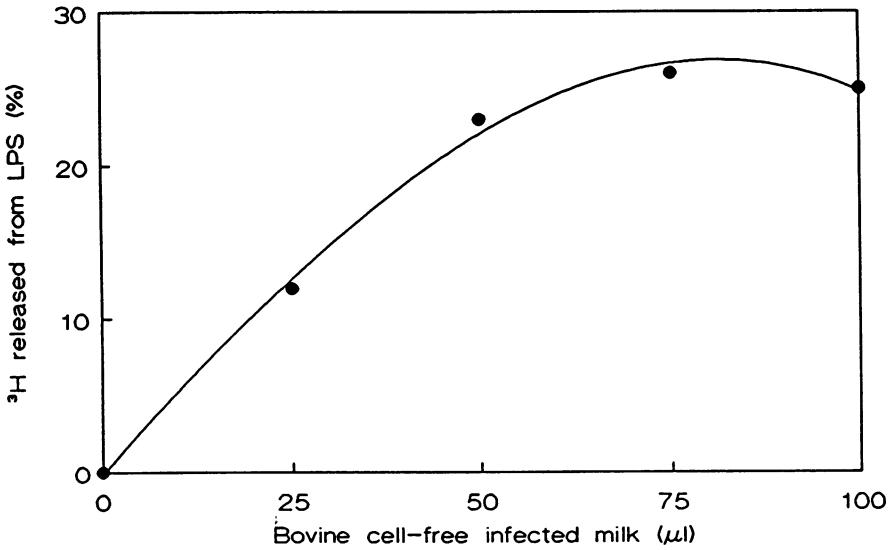

FIG. 4. Analysis of the effect of varying enzyme concentration on reactivity with substrate. Increasing amounts of bovine cell-free infected milk were added to a constant quantity of radiolabeled LPS, and ^{3}H counts released were measured following incubation.

an absolutely (100%) pure population of neutrophils from milk, we have referred to our isolated cells as leukocytes. In noninfected milk, cell populations consist of 66% neutrophils, 16% lymphocytes, 10% monocytes-macrophages, and 8% epithelial cells. During infection by gram-negative bacteria, these cell populations change to 99% neutrophils and a combination of other cells equaling 1%. Because this experiment was designed to study acute infection and was conducted for only 72 h postinfection, there were no changes in cell populations toward mononuclear cell (monocyte-macrophage) accumulation. Although monocytes may be responsible for the AOAH activity detected in bovine milk, their small number and their percent decrease during infection make this possibility less likely. It appears that neutrophils are the cell population in bovine milk responsible for the increases in AOAH activity measured in cell lysates.

The possibility exists that there is an innate difference between the stability of neutrophils isolated from sites of

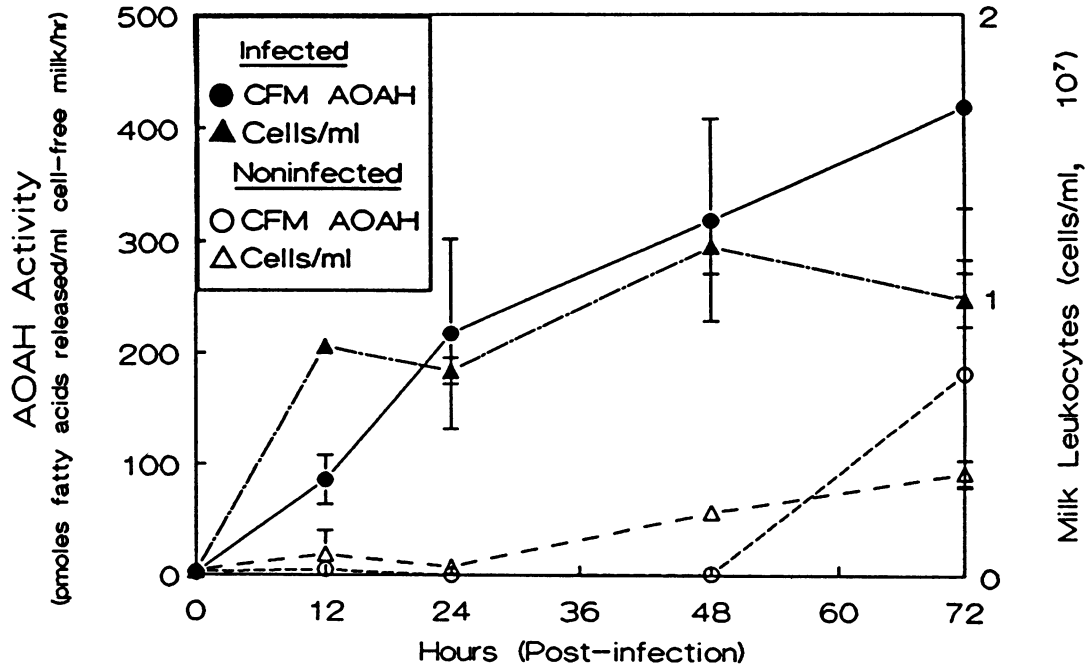

FIG. 5. AOAH activity in bovine cell-free infected and noninfected milk. AOAH activity was measured in cell-free milk (CFM) collected prior to and during intramammary gram-negative bacterial infection. Triangles represent leukocyte numbers measured in infected and noninfected milk before centrifugation. Error bars represent the standard errors of the means for seven observations. Increasing AOAH activity in infected cell-free milk corresponds with increases in leukocyte numbers.

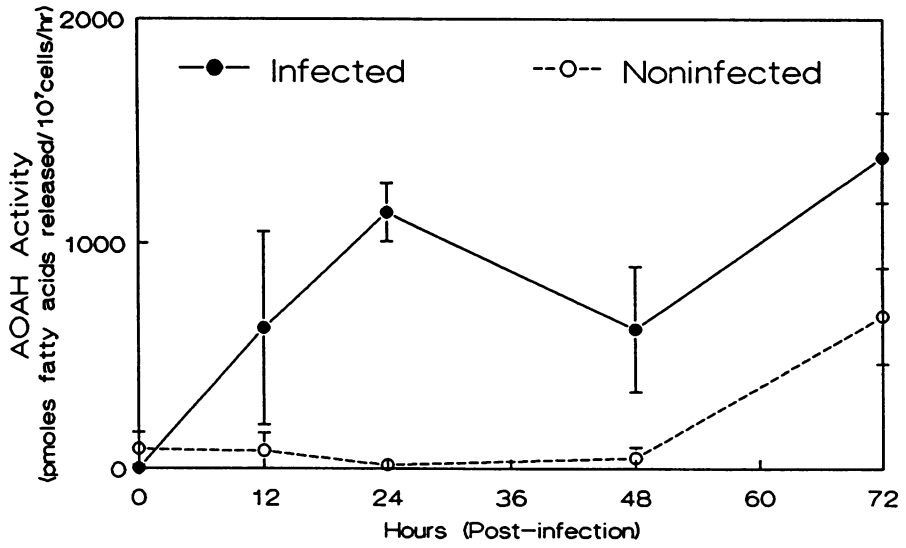

FIG. 6. AOAH activity in bovine mammary leukocyte lysates from infected and noninfected mammary glands. AOAH activity was measured in bovine mammary leukocytes prior to and during intramammary gram-negative bacterial infection. Error bars represent the standard errors of the means for seven observations. At all measured time points following infection, the infected samples had significantly higher AOAH activity than noninfected samples ($P < 0.05$).

high neutrophil numbers (infected) and those isolated from sites of low neutrophil numbers (noninfected). Therefore, a difference would exist in the ability to measure neutrophil lysate AOAH activity from infected versus noninfected mammary glands. However, all cells were handled in an identical manner to minimize this potential discrepancy. In addition, conditions at sites of inflammation (high neutrophil numbers) were such that these cells would be more likely to have undergone degranulation or secretion of lysosomal products than would neutrophils from noninfected glands (low neutrophil numbers). Neutrophils from infected glands possessed the ability to deacylate LPS to a greater degree than neutrophils from noninfected glands. Innate differences in neutrophil stability were effectively eliminated in this study. There is also the possibility that neutrophils from different locations or environments differ in their ability to bind endotoxin, as has been found among leukocytes from different species (18). Furthermore, while it is clear that AOAH activity increased in association with infection, it is unknown if this increase is due to increased production or increased activity of a single enzyme or possibly the production of a new enzyme(s) with a similar function.

We found evidence of an inhibitor(s) of AOAH activity in bovine cell-free milk (unpublished data) and postulated that this inhibitor may have a role in our inability to measure significant enzymatic deacylation of LPS by leukocytes isolated from noninfected milk. The inhibitor is present in both infected and noninfected milk, however, and infected milk inhibits deacylation of LPS to a greater degree than does noninfected milk. This finding lends credibility to the

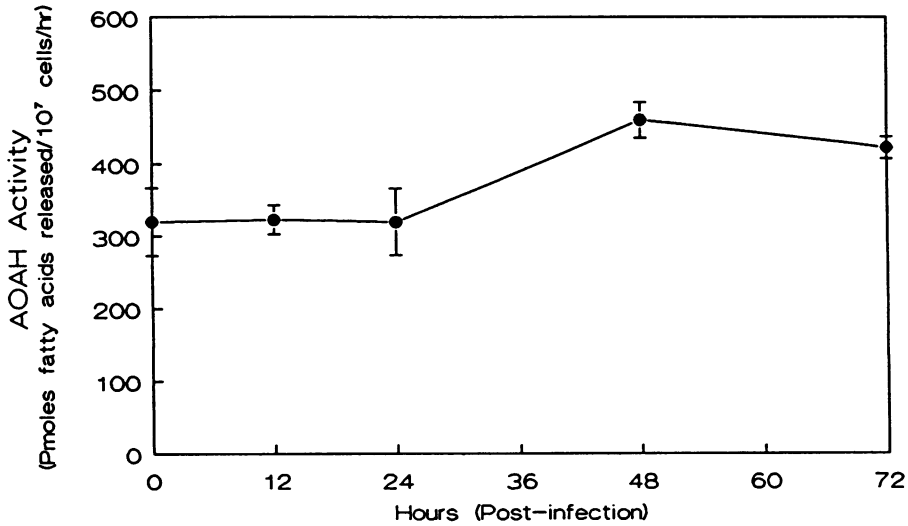

FIG. 7. AOAH activity in bovine peripheral blood leukocytes following localized gram-negative bacterial infection. Error bars represent the standard errors of the means for seven observations.

observations of this study in that even in the face of increasing inhibition, AOAH activity rose in the cell-free infected milk during inflammation. While the pH decreases slightly during the early stages of coliform mastitis, the change was not sufficient to significantly alter the measured activity based on the influence of pH on activity that was measured in vitro.

Leukocytes (and cell-free milk) isolated from noninfected mammary glands showed increased AOAH activity per cell at 72 h postinfection (Fig. 5). This increase in enzyme activity corresponds to a sterile inflammatory reaction noted during this same time period. Although an inflammatory reaction in a noninfected gland adjacent to an infected gland is frequently observed in bovine mammary infections (19), the mechanism by which this occurs is not well understood. Perhaps increased circulating endogenous inflammatory mediators reach other noninfected glands via systemic circulation or perhaps collateral vascular beds are opened by vasodilatory substances of inflammation and allow locally recruited inflammatory cells to reach noninfected glands.

Leukocytes isolated from circulating blood over the course of infection contain slightly increased AOAH activity per cell (Fig. 7). This may be attributable to a slow release of endogenous mediator(s) from the localized infection to the systemic circulation or to changes in circulating leukocyte subpopulations. The inability to measure AOAH activity in bovine plasma may have been due to assay insensitivity, lack of release of AOAH from peripheral blood leukocytes into plasma, or the presence of an inhibitory substance(s) in plasma which does not allow AOAH activity to be measured in our assay system. These increases do not appear to be the result of experimental manipulations, as previous experiments involving noninfected cows, treated in essentially the same fashion, showed insignificant fluctuations in leukocyte or milk AOAH activity over a 2-week period (data not shown).

LPS, being a component of the gram-negative bacterial cell wall, is released into the extracellular environment during infection, where it interacts with macrophages, platelets, and other cells of the inflammatory cascade, stimulating production of a variety of endogenous mediators (3, 4, 10). These mediators are responsible for a large portion of the clinical events that occur during gram-negative infection and inflammation (10, 11).

From the data presented here in combination with our preliminary data suggesting that AOAH activity may be increased in leukocytes following any inflammatory event (not only following gram-negative infection), we hypothesize that the AOAH activity increase in leukocytes may be stimulated by an endogenous mediator(s). Activation of leukocytes may be involved with increases in AOAH activity, or on the other hand, changes in leukocyte subpopulations may be responsible. A combination of the two hypotheses is also feasible.

Regardless of whether increased AOAH activity involves leukocyte activation or changes in leukocyte subpopulations, the importance of de novo protein synthesis in AOAH activity increase is unknown. Several neutrophil enzymes are stored in an inactive form and are chemically altered and thus activated when required (20, 21). Granulocyte-macrophage colony stimulating factor increases the superoxide anion-producing ability of neutrophils, and this increased production is reduced if protein synthesis is blocked (22). The enzyme responsible for superoxide anion production has activity that is low in resting cells but greatly increases when cells are appropriately stimulated. This finding suggests an important role for de novo protein synthesis in enzyme activity in neutrophils.

The detection of AOAH activity in the cell-free fraction of mammary secretions indicates that, in the cow, the enzyme may function outside of the lysosome. The appearance of AOAH activity in the extracellular fluid of the bovine mammary gland, however, does not elucidate whether the enzyme is released from leukocytes as they are lysed or is a secretory product of viable leukocytes.

Acyloxyacyl-linked fatty acids of lipid A are necessary for the production of certain mediators by macrophages (2). In addition, deacylated LPS inhibits in vitro neutrophil-endothelial cell interactions specifically induced by LPS (17), thus competing with native (acylated) LPS for interaction with target cells. Localization of AOAH activity solely within the lysosomes of neutrophils, without the ability to function extracellularly, would eliminate many potential benefits the host would derive from this enzyme. Detection of AOAH activity in the extracellular milieu suggests that AOAH may have a role in detoxification of LPS before phagocytosis occurs and, thus, in mediating the consequences of endotoxin.

Knowledge of how endotoxin is catabolized and detoxified may be a step toward development of a novel therapy against gram-negative infection. Although antibiotics are often effective in killing the organism, many clinical signs observed during gram-negative infection can be attributed to endotoxin and host defense mechanisms triggered by endotoxin. Further study exploring the deacylation of lipid A by AOAH is warranted and may eventually result in use of AOAH as a prophylactic or therapeutic agent during gram-negative infection. In addition, this work may help explain why neutrophils, at least in the bovine, provide a degree of protection from the effects of endotoxin rather than contribute to the severity of the host response (7).

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