Apoptosis of bovine neutrophils during mastitis experimentally induced with *Escherichia coli* or endotoxin

Kaat Van Oostveldt, PhD; Grant M. Tomita, PhD; Max J. Paape, PhD; Anthony V. Capuco, PhD; Christian Burvenich, DVM, PhD

**Objective**—To determine whether apoptosis of neutrophils was accelerated during mastitis experimentally induced by use of *Escherichia coli* or *E coli* endotoxin and whether differences were apparent in the response to *E coli* or endotoxin.

**Animals**—11 healthy lactating Holstein cows.

**Procedure**—Blood samples were collected from cows at various intervals after intramammary inoculation with *E coli* or endotoxin. Percentage of apoptotic neutrophils detected after in vitro incubation for 3 hours was determined. Fluorescein isothiocyanatelabeled annexin-V in combination with propidium iodide was used to distinguish apoptosis and necrosis of neutrophils. Total and differential circulating leukocyte counts and rectal temperatures were determined at the time of collection of blood samples. Milk yield and milk somatic cell counts were determined at the time of milking.

**Results**—Inoculation of endotoxin did not accelerate in vitro induction of neutrophil apoptosis. However, inoculation of *E coli* increased the percentage of apoptotic neutrophils. At 18 hours after inoculation, 20% of the neutrophils were apoptotic, compared with 5% before inoculation. Milk somatic cell count and rectal temperature increased, milk production and total leukocyte count decreased, and percentage of immature neutrophils increased after inoculation with *E coli* or endotoxin. However, kinetics of the responses were more rapid, more severe, and of shorter duration during endotoxin-induced mastitis.

**Conclusions and Clinical Relevance**—In vitro induction of apoptosis of neutrophils was accelerated only during *E coli*-induced mastitis and not during endotoxin-induced mastitis. Endotoxin inoculation as a model for studying coliform mastitis in dairy cows should be viewed with caution. (Am J Vet Res 2002;63:448–453)

Neutrophils form the first line of immunologic defense against bacteria that penetrate the physical barrier of the teat canal. Neutrophils protect the mammary gland by phagocytosis and intracellular killing of bacterial pathogens. Although neutrophils phagocytize and kill invading pathogens, they inadvertently release chemicals that induce swelling of secretory epithelium, sloughing of secretory cells, and a decrease in secretory activity. Permanent scarring will result in loss of milk production. After migration and killing of bacteria, neutrophils die as a result of apoptosis or necrosis. Apoptosis is a regulated form of cell death that limits inflammation and follows a defined process of cell shrinkage, alteration in cellular membranes, chromatin condensation, DNA fragmentation, membrane blebbing, and finally decay into apoptotic bodies and removal by phagocytosis. Resident and newly migrated macrophages help reduce the damage to the epithelium by phagocytizing neutrophils that undergo apoptosis.

Human neutrophils in all stages of apoptosis have reduced cell function, such as phagocytosis and respiratory burst activity, that may increase risk of infection. Neutrophil apoptosis is induced following diapedesis through an endothelial monolayer and ingestion of bacteria. During mastitis experimentally induced with *Escherichia coli*, circulating neutrophils have increased spontaneous respiratory burst activity 18 hours after bacterial inoculation. An inverse relation was found between respiratory burst competence and severity of coliform mastitis in cows. During *E coli*-induced mastitis, leukocytes are confronted with a number of inflammatory mediators that are expected to modulate cell function. Lipopolysaccharide content in plasma during coliform mastitis has been associated with an increase in plasma *tumor necrosis factor-α* (TNF-α). Furthermore, the concentration of TNF-α in the circulation was greater during mastitis experimentally induced with *E coli*, compared with endotoxin-induced mastitis resulting after inoculation of the same endotoxin concentration that was used in another study conducted by our research group. Recombinant TNF-α can induce in vitro apoptosis of bovine neutrophils. Intramammary infusion of endotoxin does not induce severe mastitis or a large increase in circulating concentrations of TNF-α.

In vitro, bovine neutrophils typically die through apoptosis, as determined by a flow cytometric technique that uses fluorescein isothiocyanate (FITC)-labeled annexin-V in combination with propidium iodide. Apoptosis does not occur in the circulation and is detected only in the bone marrow and in tissues. Therefore, in the study reported here, we studied apoptosis in blood incubated for 3 hours in vitro at 37°C to
evaluate the effect of treatment on sensitivity to induction of apoptosis in vitro or the progression of an apoptotic process that was stimulated in vivo.

*Escherichia coli* endotoxin may contribute to apoptosis of neutrophils. Intramammary injection of *E coli* or a fresh filtrate from an *E coli* culture produces an inflammatory response that includes damage to mammary epithelial cells. However, intramammary injection of purified commercially available *E coli* endotoxin produces an inflammatory response without damage to secretory epithelium. Furthermore, a labile toxin produced by *E coli* and not found in purified endotoxin is capable of causing epithelial damage. Similarly, addition of *E coli* culture filtrate to mammary tissue explants produces extensive epithelial cell damage, whereas damage is not observed after addition of purified endotoxin. Effects of intramammary injection of *E coli* or purified endotoxin on apoptosis of circulating neutrophils have not been investigated. However, mastitis experimentally induced with *E coli* can activate proapoptotic genes and induce apoptosis in bovine mammary parenchymal tissue.

Clinical signs of coliform mastitis are related to the quantity of cytokines released by the mammary gland and absorbed into the circulation. We hypothesized that these cytokines may initiate the apoptotic process in circulating neutrophils or sensitize these cells to other signals that would initiate cell death. Because the systemic cytokine response to intramammary infection differs from that for endotoxin treatment, we postulated that the apoptotic response to these inflammatory stimuli may differ in a similar manner (ie, lesser magnitude following endotoxin injection into the mammary gland).

The study reported here was designed to provide information about the importance of apoptosis during inflammation and, most specifically, during mastitis and to provide information about cytokines in the circulation and their ability to modulate cell function and potentially accelerate apoptosis. Differences between mastitis experimentally induced with *E coli* and endotoxin-induced mastitis on total and differential circulating leukocyte counts, milk somatic cell count (SCC), and severity of clinical signs also was compared.

**Materials and Methods**

**Animals**—Eleven healthy Holstein cows in their second to sixth lactation were selected from the USDA dairy herd in Beltsville, Md for use in the study. All cows were between 2 and 6 weeks after parturition, and none had intramammary infection with major or minor mastitis pathogens. Mean SCC for all mammary glands was 20,000 cells/ml of milk. Mean milk production was 9,000 kg/y. Use of animals for this investigation was approved by the research center’s animal care and use committee.

**Bacterial preparation and inoculation of *E coli* and endotoxin**—Six cows were randomly selected for inoculation with *E coli*, and 5 were inoculated with endotoxin. Inoculation was performed immediately after a specified morning milking. The organism used was serum-resistant *E coli* (strain P4, serotype O32:H37), which originally had been recovered from a cow with clinical mastitis and has been used in other studies of coliform mastitis. To prepare the inoculum, a tube of brain-heart infusion broth was inoculated with lyophilized *E coli* and incubated for 18 hours at 37°C. The resulting culture was streaked onto a trypticase soy blood agar plate. After incubation, several colonies were transferred from the trypticase soy blood agar plate to a fresh tube of brain-heart infusion broth, and the tube was incubated for 6 hours at 37°C. A 1-ml sample of the culture then was transferred to a flask containing 150 ml of trypticase soy broth. After incubation for 24 hours at 37°C, the flask was swirled vigorously and plunged into ice water.

On the basis of consistency of the bacterial population in cultures, the inoculum was prepared, using serial dilutions in blanks containing 99 ml of sterile pyrogen-free saline (0.9% NaCl) solution and a final fractional dilution. The desired inoculum was 250 colony-forming units/ml. Following aseptic preparation of teat ends, 500 colony-forming units of *E coli* were inoculated into each of the glands on the left side of the 5 cows, using a sterile teat cannula. The inoculated glands were gently massaged for 30 seconds to distribute the bacterial suspension. Similarly, mastitis was induced by injecting 500 μg of endotoxin (0111:B4 Sigma) in 2 ml of pyrogen-free saline solution into each of the glands on the left side of the 5 cows. Following inoculation of *E coli* and endotoxin, local and systemic variables, such as udder swelling, appearance of abnormal milk, and rectal temperature, were monitored at the time of collection of blood samples.

**Milk yield**—Total daily milk yield was obtained by combining morning and evening milk yields for the day of inoculation and days 1, 2, 3, 6, and 9 after inoculation.

**Determination of SCC**—Milk samples were obtained from weigh jars during the milking immediately preceding inoculation (time 0) and 12, 24, 48, 144, and 216 hours after inoculation. Number of SCC was determined by a fluorescent dye (ie, ethidium bromide). Samples were heated to 60°C for 15 minutes to make the cell membranes more permeable to the fluorescent dye (ie, ethidium bromide). Samples were then maintained at 4°C until counted. The cell counter was calibrated monthly, using standard concentrations of SCC.

**Collection of blood samples and leukocyte counts**—Blood samples were aseptically collected into heparin-coated tubes from an external jugular vein of each cow, using an indwelling catheter. Samples were collected at the time of bacterial or endotoxin inoculation (time 0) and 4, 8, 12, 18, 24, 48, 144, and 216 hours after bacterial or endotoxin inoculation. Total leukocyte counts were determined, using an electronic cell counter. Smears were prepared from samples of heparinized whole blood; slides were processed on an automatic slide stainer, using Wright stain. Differential microscopic counts were determined by examining 100 cells. Relative proportions of mature and immature neutrophils also were evaluated.

**Bacterial culture**—At each of the 5 morning milkings after bacterial or endotoxin inoculation, samples of foremilk were aseptically collected from inoculated and noninoculated mammary glands to assess infection status at the time of inoculation and to evaluate the establishment of infection with *E coli* in each gland. Each sample (0.05 ml) was plated onto trypticase soy blood agar for identification of *E coli* and determination of purity. Infection was defined as recovery of *E coli* or other organisms from at least 2 consecutive samples with a concurrent increase in milk SCC.

**Apoptosis of neutrophils in blood during experimentally induced mastitis—Exposed phosphatidylserine (PS) molecules on apoptotic neutrophils were stained with FITC-labeled annexin-V, and cellular DNA of necrotic cells
was stained with propidium iodide. During the early stages of apoptosis, PS is translocated from the inner face of the plasma membrane to the cell surface. On the cell surface, PS can be detected by staining with FITC-conjugated annexin-V, a protein that has a strong natural affinity for PS in a medium with highly concentrated amounts of calcium. Apoptotic cells have an intact membrane and exclude propidium iodide, whereas necrotic cells have lost their membrane integrity, and nuclei are stained with propidium iodide. The percentage of apoptotic neutrophils was quantified, using a dual-color flow cytometric procedure performed in accordance with the technique described elsewhere. Briefly, 10 µl of propidium iodide solution (50 mg/ml) and 10 ml of FITC-labeled annexin-V was added to 300 ml of incubation buffer (10 mM HEPES-NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl₂). Heparinized blood samples (100 ml) initially were incubated at 37°C under an atmosphere of 5% CO₂ and 95% air for 3 hours to promote in vitro apoptosis. An aliquot (100 µl) of the incubation buffer containing the FITC-labeled annexin-V and propidium iodide then was added to the previously incubated blood samples, and the mixture was allowed to incubate in the dark at room temperature (18°C) for 30 minutes. Individual cells were characterized on the basis of forward scatter (related to cell size) and orthogonal scatter (related to cell granularity). Dot plots were generated for gated neutrophils. Living nonapoptotic cells had negative results for FITC-labeled annexin-V and propidium iodide. Cells that had positive results for FITC-labeled annexin-V and negative results for propidium iodide were considered apoptotic, and cells that had positive results for FITC-labeled annexin-V and propidium iodide were considered necrotic or dead. Erythrocytes were lysed with 600 µl of formic acid solution (1.4 mg/L). The remaining leukocytes were stabilized with 265 ml of buffer solution (6.0 g of sodium carbonate/L, 14.5 g of sodium chloride/L, 31.3 g of sodium sulfate/L) and fixed with 100 ml of 1% paraformaldehyde before analysis on a flow cytometer.

Statistical analysis—All statistical analyses were computed in accordance with procedures described by Snedecor and Cochran, using statistical software. Results were expressed as mean ± SEM. Normality was tested, using the Wilke-Shapiro test. The Kruskal-Wallis nonparametric test for pairwise comparison was used to analyze the mean, using cow as the randomized factor, when the population was not normally distributed. When the population was normally distributed, data were subjected to an ANOVA with cow as the randomized factor, time as the fixed factor, and cow X time as the interaction. Least-significant differences were used to compare means. A value of P < 0.05 was considered significant.

Results

Bacterial culture after inoculation—Infection with E. coli was established in all of the E. coli-inoculated glands. We did not detect any accidental infections with other organisms that resulted from E. coli or endotoxin inoculation. Noninoculated glands remained uninfected throughout the study.

Clinical variables—Clinical signs of mastitis such as redness and swelling of the udder, abnormal milk, and fever were apparent in all cows after bacterial and endotoxin inoculation. However, all clinical signs were manifested earlier after endotoxin inoculation than after E. coli inoculation. Redness and swelling of the udder and the appearance of abnormal milk were detected 4 to 8 hours after endotoxin inoculation. The same signs were not detected until 12 to 18 hours after E. coli inoculation. Rectal temperature increased significantly (P < 0.001) and peaked 4 and 12 hours after endotoxin and E. coli inoculation, respectively (Fig 1). Endotoxin inoculation resulted in a more rapid and greater increase in rectal temperature, compared with E. coli inoculation.

Milk yield—Milk yield for E. coli- and endotoxin-inoculated cows decreased significantly the day after inoculation (Fig 2). By day 3 after inoculation, milk yield for E. coli-inoculated cows returned to the yield obtained before inoculation. However, milk yield for endotoxin-inoculated cows remained less than the yield obtained before inoculation throughout the remainder of the study.

Milk SCC—The SCC began to increase in all inoculated glands 12 hours after inoculation (Fig 3). Peak response was observed 12 hours after endotoxin inoculation and 24 hours after E. coli inoculation, at which
time SCC were 40.0 × 10^6 and 1.0 × 10^6 cells/ml, respectively. Milk SCC for all inoculated glands returned to preinoculation concentrations by 144 hours after inoculation.

**Total and differential leukocyte counts—**Four hours after endotoxin inoculation, total leukocyte counts already were decreased (Fig 4). At 8 hours, leukocyte counts were significantly decreased (P < 0.001) and at their lowest values, but they returned to preinoculation values by 48 hours after inoculation; they then increased again and significantly (P < 0.05) exceeded preinoculation values 216 hours after inoculation. After *E coli* inoculation, total leukocyte counts started to decrease by 8 hours; counts were significantly (P < 0.001) decreased and lowest 12 hours after inoculation but returned slowly to preinoculation values by 48 hours after inoculation. When total leukocyte counts decreased, there was a concomitant and severe decrease in the percentage of mature neutrophils and an increase in the percentage of immature neutrophils in each inoculation group. Percentage of mature neutrophils decreased significantly, and percentage of immature neutrophils increased significantly 4 hours after endotoxin inoculation, but they returned to preinoculation values by 48 hours after inoculation. Percentage of mature neutrophils decreased significantly and percentage of immature neutrophils increased significantly 12 hours after *E coli* inoculation. Percentage of immature neutrophils peaked 24 hours after inoculation and returned to preinoculation values 216 hours after inoculation. Changes in percentage of lymphocytes were not detected following endotoxin inoculation. Percentages of monocytes and eosinophils remained unchanged throughout the study for both inoculation groups (data not shown).

**Apoptosis of neutrophils after *E coli* or endotoxin inoculation—**Approximately 5% of the neutrophils in blood samples obtained at the time of inoculation were apoptotic after in vitro incubation for 3 hours (Fig 5). Significant changes were not detected after endotoxin inoculation; however, the percentage of apoptotic neutrophils was significantly (P = 0.01) increased after *E coli* inoculation and peaked 18 hours after inoculation. Approximately 20% of the neutrophils were apoptotic in samples obtained 18 hours after inoculation that were then incubated for 3 hours. The percentage of apoptotic neutrophils returned to preinoculation values at 144 hours after inoculation. The percentage of necrotic cells remained < 5% throughout the study.
Discussion

Migration of large numbers of neutrophils from the blood stream into the mammary gland is the major cellular event at the onset of acute mastitis. Contents of neutrophil granules released at the site of infection are able to damage and kill invading bacteria, but they also can amplify the inflammatory response by releasing histotoxic contents. A resolution of localized inflammation necessitates removal of neutrophils. Human neutrophils, obtained from blood and then aged in culture, undergo morphologic changes and DNA fragmentation characteristic of apoptosis. In vivo, apoptotic neutrophils are eliminated by macrophages, immature phagocytes, fibroblasts, and epithelial cells. Throughout the entire process of apoptosis and clearance of apoptotic cells, cell membranes of neutrophils remain functionally intact, preventing release of their proinflammatory and histotoxic contents. Fadok et al documented that recognition and removal of apoptotic cells are mediated by expression of PS on the external surface of apoptotic cells and its recognition by a PS receptor on phagocytes. Additionally, the PS receptor appears to mediate production of transforming-growth factor-α and down-regulation of inflammatory cytokines, including TNF-α. Thus, apoptosis and clearance by phagocytes would assist in resolving inflammation of the mammary gland without damage to mammary gland secretory tissue. In contrast, release of toxic cellular contents when a cell dies as a result of necrosis intensifies the inflammatory reaction and causes injury to nearby cells.

To investigate whether mastitis reduces the viability of circulating neutrophils, mastitis was induced by intramammary injection of viable E coli or sterile E coli endotoxin. Intramammary injections of purified endotoxin commonly are used to simulate mastitis attributable to infection with E coli. The popularity of this technique is based on the rapid recovery of milk yield in injected mammary glands. However, whether it is appropriate to use purified endotoxin to simulate coliform mastitis has been challenged. The response of mammary gland tissues in lactating cows following injection of purified endotoxin or culture filtrates of E coli produces an acute inflammatory response, but only the culture filtrate produces damage to epithelial cells. This effect was ascribed to a cytotoxic agent released during growth of the bacteria in culture that is not contained in purified preparations of endotoxin. It also has been reported that the endotoxin model for induction of mastitis is only partially comparable to E coli-induced mastitis.

The study reported here revealed differences in kinetics of circulating neutrophils, total leukocyte counts, milk SCC; milk yield, and rectal temperature between the 2 inoculation groups. If endotoxin mediates these responses, differences between the groups may have resulted from differences in exposure of neutrophils to endotoxin as a result of divergence in the magnitude and kinetics of an increase in endotoxin concentrations in serum between groups. Of particular importance is the enhancement of apoptosis by E coli-induced mastitis but not by endotoxin-induced mastitis. Cows inoculated with E coli had a significant increase in apoptosis in samples obtained 18 and 24 hours after inoculation that were then incubated in vitro for 3 hours. In samples obtained 18 hours after inoculation, up to 20% of the neutrophils were apoptotic, compared with values of 5% before inoculation.

Because most physiologic responses were greater for the endotoxin inoculation than for the E coli inoculation, the inability of endotoxin to induce apoptosis cannot be attributed to insufficient endotoxin exposure. Inherent differences between the 2 models for coliform mastitis must be invoked. In addition to E coli-induced production of unidentified factors that may mediate this response, 2 known mechanisms may be involved. First, increased respiratory burst activity of circulating neutrophils during E coli-induced mastitis could induce apoptosis. In humans, neutrophil apoptosis was induced following neutrophil respiratory burst. To our knowledge, there have not been any reported studies on oxidative burst activity of circulating neutrophils following intramammary injection of endotoxin. Second, during initiation of the inflammatory reaction, mononuclear leukocytes and epithelial cells play an important role by secreting cytokines in response to endotoxin released by invading bacteria. These cytokines are absorbed into the circulation within the mammary gland. Tumor necrosis factor-α secreted by macrophages is one of the cytokines known to accelerate neutrophil apoptosis. During E coli-induced mastitis, TNF-α appeared in the circulation between 8 and 16 hours after inoculation, whereas after intramammary injection of 500 µg of endotoxin, it was not detected in blood or detected only at low concentrations, compared with concentrations for E coli-induced mastitis. These differences may explain the lack of apoptosis of circulating neutrophils after endotoxin inoculation. During mastitis induced by inoculation with E coli and endotoxin, neutropenia was detected, indicating that bone marrow granulopoiesis had increased. The percentage of necrotic and apoptotic cells remained constant only after endotoxin inoculation. Because apoptosis and necrosis do not occur in circulating neutrophils but only in neutrophils in the bone marrow and tissue sites such as the mammary glands, induced apoptosis of circulating neutrophils in vitro can provide suggestive evidence of sus-
ceptibility of neutrophils to apoptosis within the mammary glands. Further investigation of neutrophil apoptosis in bone marrow and milk during mastitis is needed to determine whether apoptosis is related to the severity of mastitis.

Apoptosis of blood neutrophils is accelerated in humans with acquired immunodeficiency syndrome and during bronchiolitis induced by respiratory syncytial virus. To our knowledge, this is the first study to document accelerated apoptosis of blood neutrophils in response to bacterial infection.

Apoptosis of blood neutrophils was accelerated during E. coli-induced mastitis but not during endotoxin-induced mastitis. Kinetics of circulating leukocytes, mastitis and should be viewed with caution.

References

4. Endotoxin O111:B4, Sigma Chemical Co, St Louis, Mo.
5. Trypticase soy agar plate, Remel, Lenexa, Kan.
6. Trypticase soy broth, Baltimore Biological Laboratories, Cockeysville, Md.
8. Trypticase soy broth, Baltimore Biological Laboratories, Cockeysville, Md.
9. Endotoxin O111:B4, Sigma Chemical Co, St Louis, Mo.
10. Fluorescein isothiocyanate-labeled annexin-V, Boehringer Mannheim GmbH, Mannheim, Germany.
11. Propidium iodide, Molecular Probes, Eugene, Ore.
12. EPICS Profile II flow cytometer, Coulter Electronics, Hialeah, Fla.