Expression of CD3 and CD11b antigens on blood and mammary gland leukocytes and bacterial survival in milk of cows with experimentally induced Staphylococcus aureus mastitis

Ariel L. Rivas, DVM, PhD; Fred W. Quimby, VMD, PhD; Ozden Coksaygan, MD; Argia Alba, BS; Ainhoa Arina, BS; M. Juncal Arrobas, BS; Rubén N. González, DVM, PhD; Hussni O. Mohammed, BVSc, PhD; Donald H. Lein, DVM, PhD

Objectives—To differentiate early (1 to 8 days) from late (9 to 14 days) inflammatory phases and assess relationships between leukocyte phenotype and bacterial recovery in cows with Staphylococcus aureus-induced mastitis.

Animals—10 first-lactation Holstein cows.

Procedure—Blood and milk samples were collected from 4 or 6 cows before and after intramammary infusion of sterile broth or S. aureus, respectively. Flow cytometric expression of CD3 and CD11b antigens on blood and milk leukocytes, leukocyte differential counts, bacterial counts in milk, and somatic cell counts were determined longitudinally.

Results—Density of CD3 molecules decreased on blood lymphocytes and increased on milk lymphocytes after infusion of bacteria. Density of CD11b molecules on lymphocytes and phagocytes and percentage of CD11b+ lymphocytes in milk increased significantly after infusion; maximum values were achieved during the early inflammatory phase. Density of CD3 and CD11b molecules on milk lymphocytes and macrophages, respectively, 1 day after inoculation were negatively correlated with bacterial recovery on day 1 and days 9 to 14, respectively. Density of CD11b molecules on milk macrophages and the ratios of phagocyte to lymphocyte percentages and polymorphonuclear cell to macrophage percentages in milk differentiated the early from the late inflammatory phase.

Conclusions and Clinical Relevance—Activation of bovine mammary gland macrophages and T cells in response to intramammary infection of S. aureus was associated with an inability to culture this bacterium from milk. Identification of specific inflammatory phases of S. aureus-induced mastitis in cows may allow for the design of more efficacious treatment and control programs.

Mastitis is a major health problem in the dairy industry and a relevant model for the study of local immune processes. To date, antibiotic therapy and vaccination programs against Staphylococcus aureus, a major bacterial pathogen associated with mastitis in cows, have failed to result in consistent protection. Thus, current approaches for prevention of S aureus-induced mastitis also focus on optimization of mammary gland immune responses.

Inflammatory processes involve a series of changes that include at least 3 steps. First, blood leukocytes adhere to the endothelial wall and become fully activated, after which they migrate toward the inflammatory site. Once at the site of inflammation, phagocytosis and, eventually, bacterial killing take place. These processes are mediated by selectins and integrins. The temporal assessment of activation, migration, and bacterial killing within the mammary gland is a minimal set of factors necessary to describe the host response to S aureus-induced mastitis. Although the role of mammary gland polymorphonuclear (PMN) cells has been well established in bovine mastitis, less is known about the role of mononuclear cells. The cell-surface antigens CD3 and CD11b are relevant molecules for assessment of leukocyte activation and migration processes.

Stimulation of the CD3/T-cell receptor (TCR) complex induces lymphocyte adhesion and transendothelial migration. Density of membrane-bound CD3 molecules reflects the degree of T-cell activation. On stimulation of the CD3/TCR complex, T cells are induced to adhere and migrate in response to various chemoattractants and cytokines such as tumor necrosis factor α (TNFα). The percentage of T cells in bovine milk has been assessed. However, to our knowledge, relationships between CD3 expression (ie, density of CD3 molecules/cell), lymphocyte migration (ie, percentages of blood vs mammary gland lymphocyte subtypes), and bacterial growth (ie, number of bacteria recovered after inoculation) have not been reported.

Another molecule relevant in bacteria-induced inflammatory processes is CD11b, which together with CD18 forms complement receptor 3 (CR3) or Mac-1. This cell-surface antigen is constitutively expressed on phagocytes, T- and B-cell subsets, and natural killer and cytotoxic cells in several species. On lymphocytes, CD11b is required for adhesion to and migration through the endothelium. In addition, it mediates adhesion to some parasites. On phagocytes, CD11b mediates diapedesis through the endothelium via generation of a high-affinity binding site for intercellular
adhesion molecule-1. On phagocytosis, CR3 mediates phagocytic and degranulation responses to bacteria or immune complexes opsonized with inactivated C3b, a component of the alternative complement pathway. Most of the CD11b contained in leukocytes is not expressed on the membrane but stored in intracytoplasmic granules. On stimulation with cytokines (eg, interleukin-1, TNFα), cell activating agents (eg, lipopolysaccharide), bacteria, or parasites, the increase in surface expression of CD11b depends on the concentration or amount of intracytoplasmic CD11b. Increased CD11b density has been reported in inflammatory diseases. After phagocytosis of S aureus, expression of CD11b correlates with bacterial clearance, because the CD11b molecule contains multiple sites that can bind microbes.

Although extensively studied in other species, little is known about CD11b on leukocytes in cattle. This molecule has been well characterized on blood neutrophils and intraspecies cross-sectional studies have assessed the role of activation molecules in cows with naturally occurring mastitis. However, to our knowledge, no studies have been done to describe the expression of CD11b on leukocytes of cows with experimentally induced S aureus mastitis.

In other species, immunomodulatory interventions are designed on the basis of monitoring inflammatory phases. Differentiation of early and late inflammatory phases in cows with S aureus-induced mastitis may aid in the development of improved diagnostic tests and serve as a means to assess or develop new therapeutic strategies. Immunomodulation of selective inflammatory processes has been reported in a number of species with a variety of diseases in which specific cytokines are released or supplemented during specific inflammatory phases. Because cytokines induce their effects only during a certain inflammatory phase, identification of inflammatory phases is required for studies involving cytokine dynamics.

For many decades, enumeration of the somatic cell count (SCC) has been the primary means to identify cows with mastitis. More recently, bacterial culture of milk has been performed in an effort to identify etiologic agents of mastitis. However, neither of these diagnostic tools has been evaluated for their potential to distinguish the early (1 to 8 after infection) from the late (9 to 14 after infection) inflammatory phase of mastitis. The use of flow cytometry has recently been recommended for evaluation of cells involved in the etiopathogenesis of bovine mastitis.

The purposes of the study reported here were to use flow cytometric and bacteriologic methods to evaluate the use of leukocyte phenotyping and SCC for distinguishing early and late inflammatory phases of S aureus-induced mastitis and to delineate leukocyte activation and trafficking processes and assess relationships between leukocyte phenotypes and bacterial recovery in cows with S aureus-induced mastitis.

**Materials and Methods**

**Animals**—Ten first-lactation nonperiparturient Holstein cows from Cornell University dairy herds were used in this study. These cows had no history of mastitis, at least 3 consecutive tests that yielded no bacterial growth of specific pathogens from milk samples, no individual mammary gland quarter SCC > 200,000 cells/ml, and a mean of 5 consecutive SCC < 100,000 cells/ml. Cows were randomly assigned to the inoculated (n = 6) or control (4) groups. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Cornell University, and experiments were performed year-round, using 1 cow at a time, based on animal availability.

**Bacteriologic culture techniques**—Decimal dilutions (100 µl of each dilution) of the bacterial inoculum before and immediately after infusion and of each milk sample were inoculated in triplicate onto blood agar plates and incubated at 37°C for 24 hours. The number of CFU in each replicate was determined and adjusted for the dilution factor, described, and the mean CFU/ml was determined for each sample. The strain of S aureus isolated was determined by use of automated ribotyping as described. All S aureus isolates recovered from milk of inoculated cows were the same ribotype as the inoculating strain (ribotype 116-232-S3). Somatic cell counts in milk samples from individual mammary gland quarters were determined in duplicate, using a cell counter at the North East Dairy Herd Improvement Association, and the mean SCC for each quarter on each day was calculated.

**Isolation of blood and milk leukocytes**—Blood leukocytes were isolated by use of gradient centrifugation and washed 3 times as described. Milk leukocytes were isolated by use of a modified version of the protocol described by Schmaltz et al. Briefly, milk was diluted in an equal volume of pH 7.2 PAE buffer (PBS solution with 10% acid citrate dextrose, 20 mM EDTA, and 0.1% sodium azide) and centrifuged (350 X g) for 40 minutes at 15°C. The supernatant and fat layer were poured off, and the cell pellet was washed 3 times in PAE buffer. The washed cell pellet was resuspended in...
30 ml of Hank’s balanced salt solution (HBSS), layered on a density gradient, and centrifuged (800 x g) for 30 minutes at 15 C. Leukocytes were collected, washed 3 times in HBSS containing 10% fetal bovine serum (FBS), and resuspended in 5 ml of complete media (RPMI 1640 containing 10% FBS and 5% of a tissue culture cocktail [0.1 mM nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1X antibiotic-antimycotic mix]). Blood and milk leukocyte viability was assessed by use of trypan blue exclusion. Total milk leukocyte counts were calculated on the basis of viable cells. Milk and blood leukocyte differential counts were determined at the Clinical Pathology Laboratory at Cornell University.

**Immunophenotyping of milk and blood leukocytes—** Leukocyte phenotypes were determined by use of flow cytometry. Each leukocyte sample was analyzed with each of 3 first antibodies. Thus, 3 x 10^6 blood or milk leukocytes were resuspended in PAE buffer containing 2% rabbit serum (first wash buffer) and centrifuged at 350 x g for 10 minutes. One million cells were then transferred to each of three 12 x 75-mm polypropylene tubes containing 2% rabbit serum in PAE buffer. After 10 minutes on ice, 50 µl of the appropriate first antibody was added to each tube, and tubes were incubated for 30 minutes on ice. First antibodies used included a negative isotype control antibody (mouse IgG1 isotype) and mouse monoclonal antibodies against bovine CD3 or CD11b (IgG1 isotype) diluted 1:25 in PAE buffer containing 10% rabbit serum in PAE buffer. After incubation with the first antibody, cells were washed 3 times in first wash buffer and incubated for 30 minutes at 4 C with 100 µl of FITC-conjugated rabbit anti-mouse IgG (heavy and light chains) diluted 1:25 in PAE buffer containing 10% rabbit serum (second wash buffer). Cells were then washed 4 times with second wash buffer, fixed in 500 µl of 2% paraformaldehyde in PBS solution containing 0.1% sodium azide, and kept in darkness at 4 C until analyzed by use of flow cytometry. Cells were processed and fixed within 12 hours of collection.

Cell types were identified on the basis of forward and side scatter. Backgating was conducted on CD3+ (T cells) or CD3+ cells (non-T lymphocytes) as described. Each sample was assessed by use of a 2-step procedure that included preliminary identification of cell populations by size (forward scatter) and granularity (side scatter) followed by analysis of the fluorescence gating on each cell population’s preliminary bitmap. This procedure was repeated and the bitmap adjusted until the final bitmap corresponded to the lowest possible background fluorescence (ie, fluorescence of cells stained with anti-CD3 antibody) and the highest possible specific fluorescence (ie, fluorescence of cells stained with anti-CD3 or -CD11b). At least 40,000 cells were acquired per test to obtain enough of the cell type least represented in each sample. Data were acquired and analyzed, using commercial software. Percentage of CD3+ and CD11b+ cells and the median fluorescence intensity (MFI) of each population were determined.

**Data analysis—** Cell-surface antigen density determined for 3 days after inoculation was calculated as the MFI of CD3+ or CD11b+ cells divided by the MFI of cells stained with the negative control antibody. The net cell-surface antigen density 1 day after inoculation was calculated by dividing the MFI of CD3+ or CD11b+ cells determined on day 1 by the MFI of the respective population determined on day 0. Likewise, overall antigen density during the early phase of infection (days 1 to 8 after inoculation) was calculated by dividing the mean MFI determined during this period by the MFI determined on day 0.

Two indicators of inflammatory responses were also calculated on the basis of leukocyte differential counts determined by use of cytologic methods and flow cytometry: the ratio of percentage of milk phagocytes (ie, PMN cells and macrophages) to percentage of milk lymphocytes (PL index) and the ratio of the percentage of milk PMN cells to the percentage of milk macrophages (PM ratio).

**Determination of sensitivity and specificity—** Sensitivity and specificity of SCC, PL index, and CD11b antigen density on milk macrophages as indicators of inflammation were determined. Sensitivity was defined as the ratio of true-positive results (ie, inflammation) to all positive results and specificity as the ratio of true-negative results (ie, no inflammation) to all negative results. Because there was no standard with which to compare bacteriologic culture results, bacteriologic results were regarded as 100% sensitive and 100% specific. For SCC, PL index, and CD11b antigen density, we considered true-positive results those that yielded bacterial growth and were greater than the upper limit of the 99% confidence interval (CI) determined prior to infusion (ie, on day 0; SCC, 70 ± 10 cells/ml; PL index, 1.55; CD11b antigen density, 1.81). True-negative results were results of analyses of any sample and peaked during bacteria-negative samples from inoculated cows that were within the 99% CI determined on day 0. False-positive results were results of analyses of samples from any control cow that were greater than the upper limit of the 99% CI determined on day 0. Finally, false-negative results were results of analyses of bacteria-positive samples from inoculated cows that were within the 99% CI determined on day 0.

**Statistical analyses—** When data were available for individual mammary gland quarters, values were averaged to determine a mean value for each cow. Data were checked for nonnormal distribution, and comparisons were made over time by use of the Student t-test. In addition, correlation coefficients and confidence intervals were determined. Statistical analyses were performed, using a commercial software package. For all tests, P < 0.05 was considered significant.

**Results**

**Control group—** Mean (± SD) SCC, PL index, and CD11b density determined 1 day after intramammary gland infusion of sterile broth did not differ significantly from values determined on day 0 (SCC, 59.25 ± 45.1 vs 38 ± 17.6 ± 10^6 cells/ml; PL index, 0.65 ± 0.34 vs 1.03 ± 1.43; CD11b density, 1.43 ± 0.23 vs 1.36 ± 0.21). In addition, S aureus was not isolated from any milk sample from this group.

**Leukocyte counts in inoculated cows—** Mean total blood leukocyte count determined after intramammary gland infusion of S aureus (day 1, 1.94 ± 1.23 x 10^6 cells/ml; day 4 to 8, 1.82 ± 1.08 x 10^6 cells/ml; day 9 to 14, 1.75 ± 0.80 x 10^6 cells/ml) did not significantly differ from counts determined on day 0 (1.92 ± 0.9 x 10^6 cells/ml). However, blood PMN cell counts increased significantly after inoculation and peaked during the late inflammatory phase (9 to 14 days after infusion; Fig 1).

In contrast, mean total milk leukocyte count increased significantly at day 4 to 8 after inoculation, compared with the preinoculation value (preinoculation, 5.3 ± 1.7 x 10^6 cells/L; day 1, 23.9 ± 28.0 x 10^6 cells/L; day 4 to 8, 23.4 ± 10.6 x 10^6 cells/L; day 9 to 14, 17.1 ± 7.5 x 10^6 cells/L). Milk lymphocyte and macrophage counts were also significantly greater 4 to
8 days after inoculation, compared with preinoculation values. Although PMN cell counts in milk increased on day 1, compared with the day-0 value, this difference was not significant.

Percentage of milk lymphocytes significantly decreased after inoculation. The lowest percentage was measured on day 1 and was explained by the significant increase in percentage of milk PMN cells on this day (Fig 2). Percentages of milk macrophages and blood lymphocytes, PMN cells, and monocytes did not significantly differ over time.

Leukocyte phenotypes in inoculated cows—The combined use of light scatter and fluorescence (backgating) allowed us to identify T cells (ie, CD3+ cells) and distinguish PMN cells (CD11b+ and high granularity [side scatter]) from macrophages (CD11b+ and intermediate granularity; Fig 3). Differential counts estimated by use of flow cytometry were compared with those determined by use of cytologic methods; the ratio of phagocyte to lymphocyte counts (ie, P/L index) and PMN cell to macrophage counts (ie, P/M index) determined by use of each method were signif-
significantly correlated ($r = 0.98, P = 0.02$; Table 1). A longitudinal analysis allowed us to identify the beginning, peak, and duration of CD3 and CD11b molecule upregulation. Cell-surface CD3 density decreased on blood lymphocytes and increased on milk lymphocytes after intramammary infusion of $S$ aureus (Fig 4). In addition, percentage of CD11b$^+$ lymphocytes in milk significantly increased after inoculation as did lymphocyte antigen density; both values peaked on days 4 to 8 (Fig 5 and 6). There was a 2-fold increase in CD11b antigen density on milk PMN and at least a 3-fold increase on milk macrophages during the early inflammatory phase, compared with preinoculation values.

We compared immunophenotypes of milk and blood leukocytes during the early inflammatory phase of $S$ aureus-induced mastitis to assess whether changes in blood leukocyte phenotypes were related to those in milk. To do this, we first calculated CD11b and CD3 postinoculation indices for each leukocyte type by dividing the median CD11b or CD3 density obtained on days 1 to 8 by the median value obtained on day 0. Milk lymphocyte CD3 and milk lymphocyte, macrophage, and PMN cell CD11b postinoculation indices were $> 1$, indicating that antigen density on each cell type increased after inoculation. In contrast, blood lymphocyte CD3 and blood lymphocyte and PMN cell CD11b indices were $< 1$, indicating that antigen density on these cells decreased after inoculation (Fig 7).

Figure 3—Representative bitmaps and fluorescence curves generated during flow cytometric analysis of milk leukocytes. Leukocytes were stained with monoclonal antibodies against bovine CD3 or CD11b, and differential counts were determined on the basis of cell size (forward scatter [FSC-H]) and granularity (side scatter [SCC-H]). Analyses were performed, using milk leukocytes obtained prior to (A), at 1 day (B), 4 to 8 days (C), and 9 to 14 days (D) after intramammary infusion of 200 CFU of $S$ aureus. Fluorescence curves (bottom) were generated by determining the fluorescence intensity (FL1-H or FL1-Height) of cells within the lymphocyte gate prior to infusion (A1; CD3$^+$ lymphocytes), within the PMN cell gate on days 4 to 8 (C1; CD11b$^+$ PMN cells), and within the macrophage gate on days 9 to 14 (D1; CD11b$^+$ macrophages). The No. of dots within each gate represents the No. of cells within each leukocyte subset.
detected a significant negative correlation \( (r = -0.999) \) between CD11b density on blood lymphocytes and that on milk lymphocytes over time (Fig 8). In contrast, the blood monocyte CD11b postinoculation index was > 1, indicating that antigen density increased on monocytes following intramammary infusion of \( S. aureus \) (Fig 7). However, when data for individual cows were analyzed, CD11b density on blood monocytes increased in only 3 of the 6 inoculated cows.

Relationships between leukocyte phenotypes, bacterial counts, and SCC—Density of CD11b molecules on milk lymphocytes was positively associated \( (r = 0.44) \) with that on milk macrophages. When data obtained at all 3 times after inoculation (ie, day 1, days 4 to 8, days 9 to 14) were averaged, slight but not significant negative correlations were detected between CD11b antigen density on blood and milk lymphocytes \( (r = -0.80) \), macrophages \( (r = -0.82) \), and PMN cells \( (r = -0.39) \).
Mean P/L index during the early inflammatory phase was positively correlated (r = 0.79) with the percentage of CD11b + milk lymphocytes. However, this correlation was not significant (P = 0.06). In addition, significant associations were not detected between SCC and CD11b density on milk macrophages or PMN cells at any time after inoculation.

On day 1, mean CD11b density on milk PMN cells was negatively associated with bacterial counts. However, this association was not significant. In contrast, CD3 density on milk T cells on day 1 was significantly associated (r = -0.85, P = 0.03) with mean bacterial count on day 1, and CD11b density on milk macrophages on day 1 was significantly associated (r = -0.84, P = 0.04) with bacterial count on days 9 to 14 (Fig 9).

No bacteria were recovered from milk samples from 2 of 6 inoculated cows (Table 2; cows A and B).

However, the percentage of CD11b + milk phagocytes and the density of CD11b and CD3 molecules on milk phagocytes and lymphocytes, respectively, were significantly higher in these 2 cows, compared with the remaining 4 cows. On day 1, milk lymphocyte CD3 postinoculation indices for cows A and B were greater than the upper limit of the 99% CI determined for the same index in the remaining cows. Similarly, milk macrophage and PMN cell CD11b indices for these 2 cows on day 1 were greater than the upper limit of the 91 and the 99% CI for each index, respectively, in the remaining cows.

Milk samples from 3 cows (C, E, and F) yielded the highest bacterial counts, and CD11b expression on milk lymphocytes peaked at a later time in these cows (after day 4), compared with cows A and B (day 1). These results suggest that time and CD antigen density rather than antigen density alone were associated with bacterial clearance.
On day 1, density of CD11b molecules on milk phagocytes was greater in cows A and B, compared with all other cows. At the same time, SCC in the infused quarters of these 2 cows were less than SCC in the remaining 4 cows (Table 2). Somatic cell counts also remained lower in cows A and B, compared with the other cows, at days 4 to 8 and 9 to 14.

The P/M index in milk was significantly different among cows (Table 2). The lower limit of the 95% CI for the day-1 P/M index determined for the 4 cows from which bacteria were recovered (3.46) was greater than that for cows A (3.20) and B (2.00). This suggests that the local (ie, mammary gland) immune response in cows from which bacteria were recovered was characterized by a greater number of PMN cells, compared with the response of cows from which no bacteria were recovered which was characterized by a greater number of macrophages. During the late inflammatory phase (day 9 to 14), P/M indices for cows A and B were 0.06 and 0.01, respectively, indicating that macrophages predominated in these cows, whereas indices for the remaining cows were > 0.76.

### Differentiation between early and late inflammatory phases

The early inflammatory phase of *S aureus*-induced mastitis (days 1 to 8 after inoculation) was characterized by an increase in CD11b anti-

### Table 2—Local inflammatory responses induced by infusion of 200 CFU of *Staphylococcus aureus* into the left hind (LH) and right front (RF) mammary gland quarters of 6 first-lactation Holstein cows

<table>
<thead>
<tr>
<th>Day*</th>
<th>Bacterial count (CFU/ml)</th>
<th>SCC (X 10^3/ml)</th>
<th>P/L index†</th>
<th>P/M index†</th>
<th>CD11b‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21§</td>
<td>21§</td>
<td>0.25</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>403§</td>
<td>403§</td>
<td>12.07</td>
</tr>
<tr>
<td>4–8</td>
<td>0</td>
<td>0</td>
<td>420§</td>
<td>420§</td>
<td>29.12</td>
</tr>
<tr>
<td>9–14</td>
<td>0</td>
<td>0</td>
<td>655</td>
<td>655</td>
<td>8.02</td>
</tr>
<tr>
<td>Cow B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>58</td>
<td>27</td>
<td>0.45</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>713</td>
<td>27</td>
<td>1.31</td>
</tr>
<tr>
<td>4–8</td>
<td>0</td>
<td>0</td>
<td>193</td>
<td>78</td>
<td>11.65</td>
</tr>
<tr>
<td>9–14</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>41</td>
<td>0.26</td>
</tr>
<tr>
<td>Cow C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>47</td>
<td>47</td>
<td>0.23</td>
</tr>
<tr>
<td>1</td>
<td>680</td>
<td>2,470</td>
<td>25</td>
<td>22</td>
<td>2.47</td>
</tr>
<tr>
<td>4–8</td>
<td>33,290</td>
<td>150</td>
<td>8,013</td>
<td>4,946</td>
<td>1.63</td>
</tr>
<tr>
<td>9–14</td>
<td>100,000</td>
<td>860</td>
<td>1,803</td>
<td>1,103</td>
<td>0.95</td>
</tr>
<tr>
<td>Cow D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>18</td>
<td>0.59</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>61</td>
<td>6.47</td>
</tr>
<tr>
<td>4–8</td>
<td>4,840</td>
<td>530</td>
<td>80</td>
<td>101</td>
<td>3.30</td>
</tr>
<tr>
<td>9–14</td>
<td>2,320</td>
<td>490</td>
<td>1,026</td>
<td>2,909</td>
<td>3.42</td>
</tr>
<tr>
<td>Cow E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>66</td>
<td>122</td>
<td>0.30</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>210</td>
<td>503</td>
<td>283</td>
<td>20.96</td>
</tr>
<tr>
<td>4–8</td>
<td>3,740</td>
<td>2,530</td>
<td>7,547</td>
<td>1,407</td>
<td>18.64</td>
</tr>
<tr>
<td>9–14</td>
<td>870</td>
<td>10,000</td>
<td>7,547</td>
<td>2,661</td>
<td>9.31</td>
</tr>
<tr>
<td>Cow F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>118</td>
<td>25</td>
<td>0.33</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>0</td>
<td>1,407</td>
<td>463</td>
<td>8.93</td>
</tr>
<tr>
<td>4–8</td>
<td>10,000</td>
<td>2,270</td>
<td>11,500</td>
<td>71</td>
<td>34.81</td>
</tr>
<tr>
<td>9–14</td>
<td>10,000</td>
<td>0</td>
<td>10,000</td>
<td>10</td>
<td>2.92</td>
</tr>
</tbody>
</table>

*Data reported as 90% confidence intervals (CI). †Data reported as 80% CI. SCC = Somatic cell count. ND = Not determined.

### Table 3—Phenotypic properties of milk leukocytes elicited during the early (1 to 8 days) and late (9 to 14 days) inflammatory phases of *S aureus*-induced mastitis in cows

<table>
<thead>
<tr>
<th>Property</th>
<th>Pre (n = 10)</th>
<th>Early (n = 6)</th>
<th>Late (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b density*</td>
<td>0.79–1.80</td>
<td>2.45–7.26</td>
<td>1.34–2.34</td>
</tr>
<tr>
<td>P/L index</td>
<td>0.24–0.47</td>
<td>5.15–20.07</td>
<td>1.10–7.20</td>
</tr>
<tr>
<td>P/M index†</td>
<td>ND</td>
<td>2.79–6.14</td>
<td>0.00–2.64</td>
</tr>
</tbody>
</table>

*Data reported as 90% confidence intervals (CI). †Data reported as 80% CI. CD11b = Macrophage.
lymphocytes (P/L index > 3.3 in 5/6 cows), and PMN cells predominated over macrophages (P/M index > 2.25 in 5/6 cows). During the late inflammatory phase, there was a reversal in both indices. A low P/M index (< 1 in 5/6 cows) distinguished the late from the early inflammatory phase. In contrast, SCC and bacterial counts did not differ significantly between early and late inflammatory phases (Table 1).

**Sensitivity and specificity**—Sensitivity and specificity of SCC, P/L index, and CD11b antigen density on milk macrophages as indicators of inflammation were determined. Results indicated that sensitivity and specificity of flow cytometric indicators (ie, P/L index and CD11b density) were similar to or greater than those of SCC. Sensitivity and specificity of SCC were 87.5 and 83.3%, respectively, whereas values for P/L index were 93.7 and 91.7%, respectively, and for CD11b antigen density, 93.7 and 83.3%, respectively.

**Discussion**

Inflammatory changes (ie, increased SCC and changes in leukocyte phenotypes) were detected in milk samples collected from cows that received intramammary infusions of *S aureus*. Similar changes were not detected in cows infused with the sterile vehicle alone. Thus, postinfusion inflammatory changes could not be explained by infusion of the vehicle.

Differences in mammary gland immune responses during the preinoculation, early, and late inflammatory phases of *S aureus*-induced mastitis were represented by total and differential leukocyte counts in milk samples, which were determined by use of cytologic methods. Our results, indicating that the percentage of milk lymphocytes decreased and the percentage of milk phagocytes increased after inoculation, were similar to results of previous studies.43 In addition, our results support the hypothesis that both lymphocytes and macrophages play relevant roles in the host response to *S aureus*-induced mastitis, at least during the early inflammatory phase.

Density of CD3 molecules on T cells in milk from inoculated cows reached a peak 1 day after infusion. However, peak CD11b density on lymphocytes was not detected until days 4 to 8. These findings suggest an early role for T cells in the development of the immune response to *S aureus*-induced mastitis. An increase in the ability of peripheral blood T cells to adhere to the endothelium, which is mediated by CD11b, depends on crosslinking of cell-surface CD3 molecules.42 Crosslinking of CD3 molecules facilitates T-cell adhesiveness.42 Activation of T cells through CD3 crosslinking is necessary to induce rapid and specific immune responses such as those induced by memory T cells. Activation and migration of memory T cells is rapidly induced via binding of staphylococcal antigens and mediated via cytokines such as TNFα.44,45 Up-regulation of CD3 expression, as evidenced by an increase in cell-surface density, is a central event leading to up-regulation of CD11b and, thus, T-cell activation and memory cell recruitment.

We also detected an increase in the percentage of CD11b⁺ milk lymphocytes and an increase in CD11b density on milk lymphocytes after inoculation. This further emphasizes the role of lymphocytes in the host response to mastitis. Because of the brief lifespan of phagocytes after migration into tissue, the outcome of local bacterial invasion rests on circulating T cells and differentiated monocytes.2 The relationship that we detected between the percentage of activated (CD11b⁺) milk lymphocytes and the magnitude of the inflammatory response (expressed by the P/L index) suggest that the local phagocytic inflammatory response to intramammary infusion of *S aureus* is associated with expression of the CD11b antigen on lymphocytes but is not dependent on absolute T-cell numbers. Given that we also detected a significant negative correlation between CD11b density on blood lymphocytes, which decreased after inoculation, and CD11b density on milk lymphocytes, which increased after inoculation, and given that CD11b is expressed on memory T cells in some species,2,46 we cannot rule out a selective migration of memory T cells from the blood to the site of inflammation (ie, the mammary gland).

Density of CD11b molecules on the surface of milk macrophages increased rapidly after intramammary infusion of *S aureus*. The expression of this molecule on milk macrophages increased from approximately 33% of the expression on blood monocytes immediately prior to inoculation to a level exceeding that on blood monocytes 1 day after inoculation. In humans, inflammatory macrophages are activated to a greater degree than blood monocytes.47 However, this response was brief. During the late inflammatory phase (day 9 to 14), CD11b density on milk macrophages was less than that on blood monocytes. The early and brief activation of milk macrophages appeared to differ from that of milk PMN cells; the latter cell type remained activated (high CD11b density) for a prolonged period. Cytokine differences may explain discrepancies in phagocyte activation patterns.2

In samples collected between days 4 and 8 after inoculation, CD11b expression on milk PMN cells was greater than that on blood PMN cells. This finding is consistent with results of a previous report46 indicating that after migration, CD11b density on inflammatory PMN cells may be greater than that on blood cells. The increase in CD11b expression on milk PMN cells, which occurred at the same time or later than that on milk macrophages, was consistent with previous results45,48 indicating a dichotomy between macrophages and PMN cells in that enhanced antigen expression on one cell type is independent of the expression of the same antigen on the other cell type. A significant correlation was detected between high CD3 density on milk T cells and high CD11b density on milk macrophages on day 1 and bacterial clearance. This implied that intramammary infusion of *S aureus* resulted in immediate up-regulation of CD3 antigen expression on the surface of T cells, which is in contrast to antigen-specific responses that induce T-cell differentiation. These antimicrobial effects associated with T-cell activation occurred within 24 hours of inoculation. These findings support the hypothesis that a rapid bactericidal immune response associated with T cells may develop in cows with
S aureus-induced mastitis. The γδ T-cell subset is known to exert immediate and direct antimicrobial activity, because activation of these cells does not require MHC-restricted antigen processing.19 The antimicrobial activity of γδ T cells can reduce the number of CFU of S aureus by up to 5 orders of magnitude.20 Although γδ T cells were not specifically assessed in this study, others have reported that this population of T cells increases in cows with naturally occurring staphylococcal mastitis.21 Moreover, this increase is dependent on CD4+ T cells. We have also reported an increase in number of CD4+ T cells during the early inflammatory phase of experimentally induced S aureus mastitis.37 These findings confirm the need to assess whether γδ T cells are associated with bacterial clearance in cows with mastitis.

After intramammary infusion of S aureus, expression of CD11b on milk macrophages was negatively correlated with milk bacterial counts. Bacterial binding is dependent on the expression of CD11b by macrophages,22 and crosslinking of CD11b molecules mediates nitric oxide production.23 Thus, if CD11b density on milk macrophages is high, macrophages will be able to bind and kill bacteria, and bacterial recovery from these milk samples will be low. We did detect a slight but not significant negative correlation between CD11b density on milk PMN cells and bacterial recovery. Others have reported that CD11b density on PMN cells is negatively correlated with bacterial counts.24 The difference that we detected between macrophages and PMN cells may be explained by the earlier and briefer activation peak reached by macrophages.

Analysis of data obtained for individual cows suggests that at least 2 immune profiles are possible in response to intramammary infusion of S aureus. One profile was characterized by the inability to recover bacteria from milk samples, an early and profound up-regulation of CD3 and CD11b antigens on milk leukocytes, and a low SCC and P/M index. The second profile was characterized by the ability to isolate S aureus from milk, less or later CD3 and CD11b expression, higher SCC, and a higher P/M index. A race may occur between cell activation, migration, and phagocytosis and bacterial growth in which time, in addition to up-regulation of cell-surface markers, may be a factor. These findings could lead to classification of the immune responses of individual cows on the basis of milk leukocyte immunophenotypes. In other species, the degree of CD11b expression (high vs low) has been used to identify individuals that are more or less prone to develop immune responses and inflammatory diseases have been identified on the basis of CD11b expression.25-27

Our data also suggest that phenotyping milk leukocytes differentiates the early inflammatory phase of S aureus-induced mastitis from the late phase. Determination of SCC or results of bacteriologic culture of milk did not distinguish between these 2 phases. The sensitivity and specificity of SCC as an indicator of inflammation and infection are not necessarily associated phenomena. That is, inflammation may develop in response to inoculation with microorganisms. However, if the inflammatory response is sufficient to prevent bacterial growth, then infection does not develop. This occurred in 2 of the 6 cows in the present study.

Our findings should not be construed as a general protective mechanism against infectious mastitis caused by other microorganisms. Differences associated with host responses and bacterial strains or concentrations prevent generalizations. Activated leukocytes do not necessarily kill bacteria; in other studies, PMN cells with a high degree of CD11b expression did not kill Escherichia coli or Streptococcus pyogenes. Other molecules such as insulin-like growth factor-1 may be required to achieve bacterial killing.28 Additional studies may be needed to confirm the sensitivity and specificity of results of flow cytometric-based diagnostic techniques as indicators of inflammation and infection in cows with infectious mastitis.

References
8. Muto S, Vetvicka V, Ross GD. CR3 (CD11b/CD18) expressed by cytotoxic T cells and natural killer cells is upregulated...
21. Escherichia coli and insulin-like growth factor I on opsonin receptor expression on bovine conglutinin and functions as a receptor for iC3b.
27. Ross GD, Cain JA, Lachmann PJ. Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J Immunol 1985;134:3307–3315.


