Comparison of L-selectin and Mac-1 expression on blood and milk neutrophils during experimental *Escherichia coli*-induced mastitis in cows

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Objective—To evaluate L-selectin (CD62L) and Mac-1 (CD11b) expression at the surface of blood and milk neutrophils during the early inflammatory response to *Escherichia coli*-induced mastitis in cows.

Animals—6 healthy Holstein heifers in early lactation.

Procedure—Blood and milk samples were collected before and after intramammary administration of 10^4 CFU's of *E coli* in the left mammary gland quarters. Bacterial counts and electrolyte concentrations in milk, rectal temperature, differential blood leukocyte counts, milk somatic cell counts, neutrophil viability, and the expression of CD62L and CD11b on blood and milk neutrophils were determined longitudinally.

Results—Bacteria grew during the first 6 hours after inoculation with a pronounced leukocytic influx. Coincident with neutrophil influx was an increase in $CD62L^+$ and $CD11b^+$ milk neutrophils, as well as an improved viability of milk neutrophils. The peak of the inflammatory reaction was reached approximately 12 hours after *E coli* inoculation. From that time forward, changes in CD62L and CD11b expression were opposed to each other, with a decrease in CD62L expression and an increase in CD11b expression on blood and milk neutrophils; the magnitude of the differences in CD62L and CD11b expression between blood and milk neutrophils decreased. Percentages of $CD62L^+$ and $CD11b^+$ milk neutrophils increased to percentages that were similar to blood neutrophils (ie, approx 92%).

Conclusions and Clinical Relevance—The presence of adhesion molecules on a large percentage of milk neutrophils during the acute inflammatory response, together with the changes in receptor density, suggest a major role for CD62L and CD11b in neutrophil function during coliform mastitis. (*Am J Vet Res* 2004;65:1164–1171)

Early neutrophil diapedesis into the mammary gland is considered a crucial event in the immunologic defense against *Escherichia coli*-induced mastitis.[1-3] This infectious disease is typically characterized by a massive and accelerated accumulation of neutrophils in milk. Adequate expression and sequential activation of L-selectin (CD62L) and Mac-1 (CD11b, CR3) adhesion molecules are pivotal for neutrophil recruitment from the systemic circulation into the mammary gland during the inflammatory response to mastitis. CD62L belongs to a family of adhesion proteins that binds to sialylated carbohydrate moieties on glycoproteins via a lectin-like domain and mediates the initial contact and rolling of neutrophils along the walls of the microvascular endothelium. After stimulation of neutrophils by chemoattractants, CD62L is shed from the cell surface, presumably to permit more rapid egress from the vascular bed. In contrast, the β_2-integrin subunit CD11b must be activated to enable tight binding to intercellular adhesion molecule 1 on endothelial cells.[4-5] Results of previous studies[6-8] indicate that mastitis caused by *E coli* indeed leads to diminished CD62L and upregulated CD11b densities on bovine blood neutrophils during the inflammatory response.

Adhesion molecules do not solely play a role during the migratory process; CD62L is also involved in signal transduction, leading to enhanced superoxide generation, activation of CD11b, and induction of mRNA for several cytokines.[9-11] CD11b is also involved in the mechanism of neutrophil superoxide generation[10] and in complement-mediated bacterial phagocytosis.[12-13] In addition, these molecules have been recognized as receptors for endotoxins.[14-15] Despite their major functional importance, temporal changes in CD62L and CD11b expression on neutrophils that are present at the inflammatory focus have not, to our knowledge, been examined during coliform mastitis.

Because of the opportunity of multiple sample collection from the site of infection by milking, experimental *E coli*-induced mastitis in cows provides a powerful tool to study the early local immune response during *E coli* infections. In addition, the study of *E coli*-induced mastitis in cows is important because clinical

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coliform mastitis is becoming the single most economically important form of mastitis.[16]

The aim of the study reported here was to gain insight into the progression of CD62L and CD11b expression on blood and milk neutrophils during the early stages of the inflammatory response in periparturient cows with experimental *E coli*-induced mastitis. The temporal changes in CD62L and CD11b expression were compared in blood and milk neutrophils and examined in relation to the onset of other inflammatory parameters.

Materials and Methods

Animals—In vivo experiments were performed on 6 healthy Holstein heifers between 2 and 4 weeks after parturition. The Ethical Committee of the Faculty of Veterinary Medicine at Ghent University approved the study. Cows were kept in individual stalls 5 months before the experimental trial. Only cows with milk **somatic cell counts (SCCs)** < 200,000 cells/mL in each individual mammary gland quarter and that had negative results for pathogenic bacteria on the basis of results of 2 consecutive milk bacterial cultures within 7 days prior to the experimental infection were accepted for the study. Cows were fed a daily ration of concentrate according to milk production and had free access to water and hay. Cows were milked twice daily at 7 AM and 4 PM with a 4-quarter milking machine.

Inoculation of microorganisms—Bacterial preparation (*E coli*, strain P4, serotype O32[a]) was performed as previously described.[17] Before inoculation, the teat ends were disinfected with 70% ethanol containing 0.5% chlorhexidine. On day 0, 1 hour after milking, a group of 6 cows was inoculated in the left front and left hind mammary gland quarters with 20 mL of a 10^4 **CFU** suspension of *E coli* in saline (0.9% NaCl) solution. Bacteria were injected through the streak canal by use of a syringe fitted with a sterile teat cannula.[b] Immediately after inoculation, each mammary gland quarter was massaged for 30 seconds to homogeneously distribute the bacterial suspension in the gland.

Sample collection—Evacuated tubes coated with heparin were aseptically filled with blood samples that were obtained from the external jugular vein by venipuncture.[c] Milk samples that were obtained from individual mammary gland quarters were aseptically collected. Blood samples were obtained daily at 1 hour after the morning milking on days 7, 4, and 1 before inoculation and on days 1, 2, 3, and 6 after inoculation. Milk samples were collected during the morning milking before blood sample collection. On the day of inoculation (day 0), blood and milk samples were collected every 3 hours. At the time of blood sample collection, rectal temperature and other clinical signs were monitored.

Milk bacterial culture and electrolyte concentrations—Milk samples were aseptically collected to verify the absence of infection before inoculation and evaluate the progress of experimental *E coli* infection in the inoculated mammary gland quarters. The amount of bacteria was determined by appropriate 10-fold dilutions of the sample in PBS solution. Each dilution was plated on blood agar plates[d] and incubated at 37°C for 24 hours. The number of CFUs in each replicate was determined and adjusted for the dilution factor; the mean CFUs per milliliter was calculated for each sample. Milk Na^+, Cl^-, and K^+ concentrations were analyzed by use of ion-selective electrodes.[e]

Blood leukocyte counts and milk SCCs—Total blood leukocyte counts were measured electronically.[f] Milk SCCs were determined for samples from each individual mammary gland quarter by means of the fluoro-optoelectronics cell-counting principle.[g] Smears of cells for microscopic examination were prepared from whole blood and isolated milk cells. Plasma was added to the milk cells in a 1:1 ratio to prevent lysis during preparation of smears. Differential cell counts in blood and milk samples were determined on microscopic examination by counting 100 cells on slides of smears stained with Giemsa-quick.[h] Relative proportions of mature, band, and immature neutrophils (ie, myelocytes and metamyelocytes) in blood samples were also determined.

Determination of blood and milk neutrophil viability—Isolation of blood neutrophils was performed by use of hypotonic lysis according to Carlson and Kaneko[18] with slight modifications. Milk samples were gently mixed, diluted 60% with cold PBS solution, and further processed as described previously.[19] The suspension of isolated blood and milk neutrophils was adjusted to a concentration of 10^6 cells/mL in PBS solution. The viability of isolated milk and blood neutrophils was determined by propidium iodide (final concentration, 10 µg/mL) staining followed by flow cytometric analysis.

Immunofluorescence labeling of neutrophils—Blood and milk aliquots of 100 µL and 1 mL, respectively, were treated with 400 µL and 4 mL, respectively, of a cold sterile buffered lysing solution (pH, 7.4) containing 21.47 mmol of Tris[i]/L and 138.34 mmol of NH_4Cl/L and gently mixed for 6 minutes. After centrifugation (200 X *g* for 10 minutes at 4°C), the cell pellet was washed twice with 300 µL of control solution containing RPMI 1640,[j] 1% albumin fraction V, and 0.2% NaN_3. The cell pellet from milk samples obtained during infection was notably increased in size, compared with milk samples obtained before inoculation. For this reason, the latter samples were diluted appropriately in control solution to obtain a cell pellet that was normal in size, which did not affect the final antibody concentration after the cells were resuspended. Subsequently, leukocytes were mixed with 100 µL of 20% bovine-pooled serum in PBS solution and 50 µL of saturating amounts of anti-bovine monoclonal antibodies that recognized CD62L (clone 11G10[k] of the IgG1 isotype[20]), CD11b (clone CC126 of the IgG2b isotype[l]), or isotype-matched controls[m] and incubated for 30 minutes at 37°C. After adding 300 µL of cold washing medium, cells were collected by centrifugation (200 X *g* for 10 minutes at 4°C). Cells were then washed once by use of the same medium before incubating them with **fluorescein isothiocyanate (FITC)**-labeled goat anti-mouse IgG as a secondary antibody (30 minutes at 4°C). Subsequently, 300 µL of PBS solution was added and cells were centrifuged (200 X *g* for 10 minutes at 4°C). All samples were subjected to a final washing with 300 µL of PBS solution, collected by centrifugation (200 X *g* for 10 minutes at 4°C), fixed in 500 µL of 1% paraformaldehyde in PBS solution, and analyzed by use of flow cytometry.

Flow cytometric analysis—Immunofluorescence measurements were performed on 10,000 cells by use of a flow cytometer.[n] The neutrophils were identified by staining with CH138A monoclonal antibodies,[o] as previously described.[19] Gates for subsequent analysis were set on neutrophils corresponding with forward and side light scatter plots of $CH138A^+$ cells. The percentage of viable neutrophils was deduced from the propidium iodide fluorescence histograms. Lack of fluorescence indicated viable neutrophils. The relative **mean fluorescence intensity (MFI)** and percentage of $CD62L^+$ and $CD11b^+$ neutrophils were calculated on the basis of the FITC fluorescence histograms after subtraction of nonspecific fluorescence. Nonspecific background fluorescence was defined as any fluorescence associated with neutrophils upon incubation with the isotype-matched controls and FITC-labeled secondary antibodies.

Statistical analysis—Analysis was based on a mixed model with cows as random effects to account for the repeat-

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ed-measures structure of the data. The first analysis was related to time 0 just before inoculation. The MFIs of blood neutrophils and percentage of $CD62L^+$ and $CD11b^+$ blood neutrophils were compared with MFIs of milk neutrophils and percentage of $CD62L^+$ and $CD11b^+$ milk neutrophils at time 0. The aim of the second analysis was to test whether the difference in MFI of neutrophils and the percentage of $CD62L^+$ and $CD11b^+$ neutrophils in blood versus milk had changed at 6 hours after *E coli* inoculation, compared with time 0. Finally, a similar analysis was done to compare the period between 12 and 48 hours with time 0. Values of $P < 0.05$ were considered significant.

Results

Clinical signs—Clinical signs of mastitis developed approximately 6 hours after intramammary inoculation. General clinical signs, such as behavioral depression and loss of appetite, were noticeable from 6 hours after *E coli* inoculation onwards. Swelling of the infected mammary gland quarters started at 6 hours and peaked between 12 and 48 hours. Flecks of blood appeared in the milk 14 hours after inoculation. At the same time, milk became watery and even purulent on day 2. Approximately 6 hours after *E coli* inoculation, milk leakage from the infected mammary gland quarters was also observed. Rectal temperature increased at 6 hours, reaching maximal temperatures at 12 hours after inoculation (data not shown).

Milk bacterial culture and electrolyte concentrations—In milk samples from the infected mammary gland quarters, the number of CFUs of *E coli* per milliliter increased immediately after *E coli* inoculation, peaking at 6 hours after inoculation and reaching concentrations of approximately 3.3×10^4 CFUs/mammary gland quarter. Six days after inoculation, only 1 cow had positive milk bacterial culture results for *E coli*.

The concentration of Na^+, Cl^-, and K^+ in milk is indicative of the integrity of the blood-milk barrier. The milk concentrations of the 3 electrolytes remained unchanged or were only slightly modified at 6 hours after *E coli* inoculation in samples obtained from the infected mammary gland quarters (**Figure 1**). Thereafter, a steep increase of milk Na^+ concentration was noticeable, peaking at 12 hours after inoculation. Milk concentrations of Na^+ then decreased gradually, but stayed approximately 23% greater than preinoculation concentrations at the end of the experimental period. The milk concentration of Cl^- closely followed Na^+ kinetics in samples from the infected mammary gland quarters. During the first 6 hours after *E coli* inoculation, the milk Cl^- concentration increased slightly, followed by an abrupt increase for up to 12 to 18 hours after inoculation. Thereafter, concentrations in milk gradually recovered to approximately 11% greater than preinoculation concentrations at the end of the experimental period. The milk K^+ concentration in samples from the infected mammary gland quarters changed in the opposite direction. From 6 hours on, milk K^+ concentrations decreased progressively, reaching minimal concentrations at 12 hours after inoculation and regaining preinoculation concentrations at 48 hours after *E coli* inoculation.

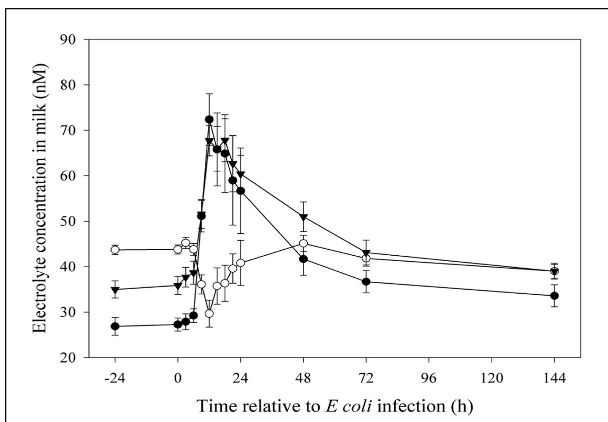

Figure 1—Mean (± SEM) concentrations of electrolytes (Na^+ [closed circle], Cl^- [closed triangle], and K^+ [open circle]) in milk versus time relative to *Escherichia coli*-induced mastitis in cows (n = 6).

Blood leukocyte count and percentage of neutrophils—Total blood leukocyte count and blood neutrophil count remained stable during the first 6 hours after *E coli* inoculation (**Figure 2**). Thereafter, total blood leukocyte and blood neutrophil counts decreased sharply. Leukopenia was pronounced, with an abrupt decrease to a minimum of 74% neutrophils at 12 hours after *E coli*

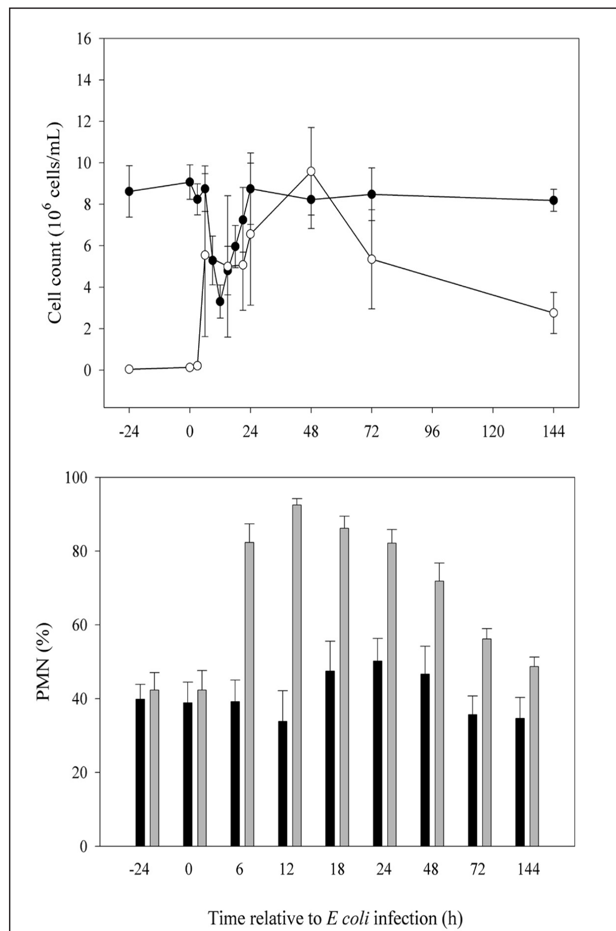

Figure 2—Mean (± SEM) total blood leukocyte cell count (top; closed circle), milk somatic cell count (top; open circle), percentage of blood (bottom; black bars) polymorphonuclear neutrophils (PMNs), and milk (bottom; shaded bars) PMNs versus time relative to *E coli*-induced mastitis in cows (n = 6).

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inoculation. Thereafter, blood leukocyte counts gradually recovered to within reference range at 24 hours.

The percentage of blood neutrophils also decreased by approximately 18% at 12 hours after inoculation, thereby actively contributing to the observed leukopenia (Figure 2). Moreover, the decrease in total numbers of neutrophils was accompanied by a decrease in mature neutrophils and an increase in the percentage of band and immature neutrophils (**Table 1**).

Milk SCCs and percentage of neutrophils—The increase in milk SCCs occurred as soon as 6 hours after *E coli* inoculation (Figure 2). Milk SCCs peaked at 24 hours after inoculation, decreasing progressively thereafter until the end of the experimental period.

The percentage of neutrophils in milk was approximately 40% before *E coli* inoculation (Figure 2). At 6 hours after inoculation, neutrophil percentages increased sharply to 80% and reached a maximum of 98% at 12 hours after *E coli* inoculation. Thereafter, the percentage decreased progressively to preinoculation values.

Viability of blood and milk neutrophils—Preinoculation milk neutrophils had a low viability of approximately 60%, whereas blood neutrophils were approximately 98% viable. The percentage of viable blood neutrophils remained unchanged during the entire experimental period. After *E coli* inoculation, viability of milk neutrophils increased sharply, reaching maximal viability percentages comparable to those in blood at 12 hours after *E coli* inoculation (**Figure 3**). Thereafter, the viability of milk neutrophils decreased progressively to preinoculation values at the end of the experimental period.

CD62L expression on blood and milk neutrophils—One hour before *E coli* inoculation, CD62L expression was significantly (*P* = 0.004) higher (on average by 39%) for blood neutrophils, compared with milk neutrophils (**Figure 4**). The difference between CD62L expression on blood and milk neutrophils remained stable until 6 hours after *E coli* inoculation, compared with control values (*P* = 0.185). Thereafter, MFIs for CD62L decreased in blood and milk neutrophils with minimal values of MFIs at 18 and 12 hours, respectively, after *E coli* inoculation, corresponding to decreases of 45% and 39%. The initial difference in CD62L expression between blood and milk neutrophils was significantly (*P* = 0.005) smaller from 12 hours until at least 48 hours after *E coli* inoculation, compared with preinoculation values. The MFIs for CD62L on blood and milk neu-

Table 1—Mean (±SEM) changes in the percentage of mature, band, and immature (ie, myelocytes and metamyelocytes) blood neutrophils during experimental *Escherichia coli*-induced mastitis in cows (n = 6).

	Neutrophils		
Time (h)	Mature neutrophils (%)	Band neutrophils (%)	Immature neutrophils (%)
–24	29.67 ± 3.84	2.67 ± 0.76	7.50 ± 0.56
0	32.00 ± 5.19	1.17 ± 0.60	5.67 ± 0.95
3	30.50 ± 3.14	3.83 ± 0.95	10.67 ± 2.26
6	18.50 ± 4.05	3.83 ± 1.76	16.83 ± 2.32
9	18.50 ± 5.64	4.00 ± 1.69	11.83 ± 1.78
12	13.50 ± 3.35	4.33 ± 1.96	16.00 ± 4.86
15	19.00 ± 3.74	8.83 ± 1.40	18.67 ± 2.20
18	19.50 ± 3.39	8.50 ± 2.14	19.50 ± 3.61
21	18.83 ± 5.14	11.83 ± 3.16	25.17 ± 3.25
24	22.67 ± 3.28	7.83 ± 1.64	19.67 ± 3.24
48	18.50 ± 3.39	5.00 ± 1.55	23.17 ± 4.63
72	12.00 ± 2.78	2.67 ± 0.99	21.00 ± 3.02
144	14.83 ± 2.76	5.33 ± 1.20	14.50 ± 2.90

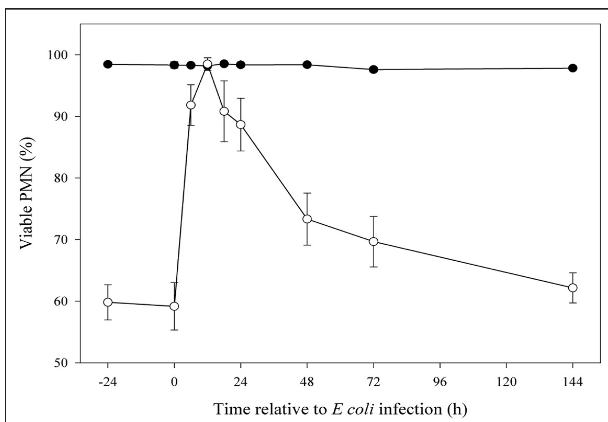

Figure 3—Mean (± SEM) percentage of viable PMNs in blood (closed circles) and milk (open circles) versus time relative to *E coli*-induced mastitis in cows (n = 6).

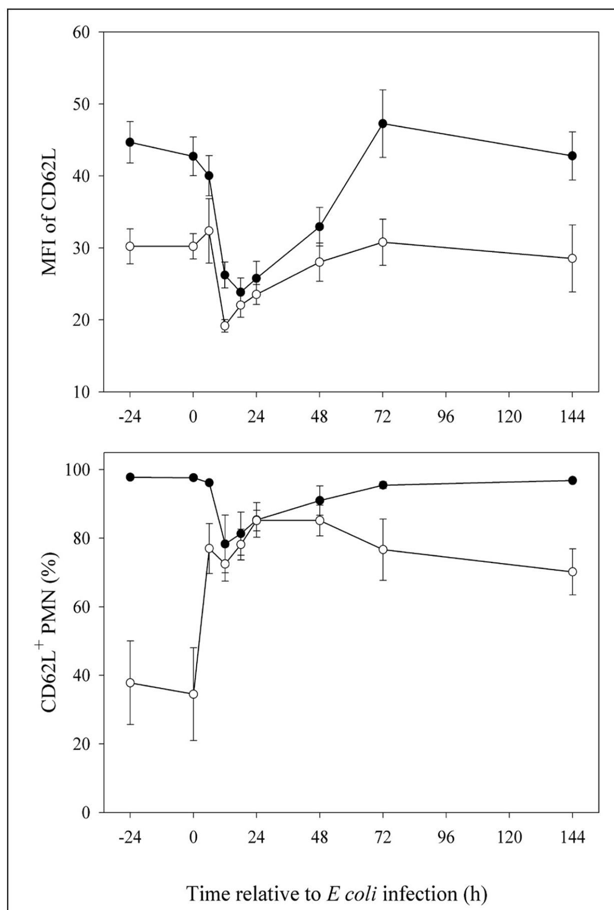

Figure 4—Mean (± SEM) values of mean fluorescence intensity (MFI) for CD62L on PMNs (top) and percentage of PMNs that were positive (+) for CD62L (bottom) in the blood (closed circles) and milk (open circles) versus time relative to *E coli*-induced mastitis in cows (n = 6).

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trophils then gradually returned to preinoculation values at 72 hours after *E coli* inoculation.

Prior to *E coli* inoculation, the percentage of $CD62L^+$ neutrophils was significantly (*P* = 0.005) lower (by 60%) in milk, compared with blood (Figure 4). Whereas the percentage of $CD62L^+$ blood neutrophils remained unaltered at 6 hours after inoculation, the percentage of $CD62L^+$ milk neutrophils increased greatly at that time. At 6 hours after *E coli* inoculation, the relative difference in the percentage of $CD62L^+$ neutrophils in blood and milk was significantly (*P* = 0.002) decreased, compared with control values. Furthermore, this decrease in the percentage between blood $CD62L^+$ and milk $CD62L^+$ neutrophils remained significant (*P* < 0.001) until at least 48 hours after *E coli* inoculation, compared with preinoculation values. The percentage of $CD62L^+$ neutrophils in blood decreased transiently to reach a minimal value at 12 hours after *E coli* inoculation, before returning gradually to preinoculation values at approximately 72 hours after *E coli* inoculation. In contrast, the percentage of $CD62L^+$ milk neutrophils increased sharply to maximal values at 24 hours after inoculation, remaining high until the end of the experimental period.

CD11b expression on blood and milk neutrophils—Preinoculation MFIs for CD11b were on average approximately 21% higher on milk neutrophils relative to blood neutrophils, but this difference was not significant (*P* = 0.057; **Figure 5**). Only minimal changes in MFI for CD11b on neutrophils were observed at 6 hours after *E coli* inoculation; likewise, the gap in CD11b expression between blood and milk neutrophils was not significantly altered at that time. Thereafter, CD11b expression started to increase on both neutrophil populations. Cell surface CD11b expression peaked at 72 hours with an increase of approximately 46% and 23% for blood and milk neutrophils, respectively, compared with values measured at the onset of the experiment. Thereafter, CD11b expression decreased gradually, and preinoculation MFIs for CD11b on blood and milk neutrophils were reestablished at approximately 144 hours after *E coli* inoculation.

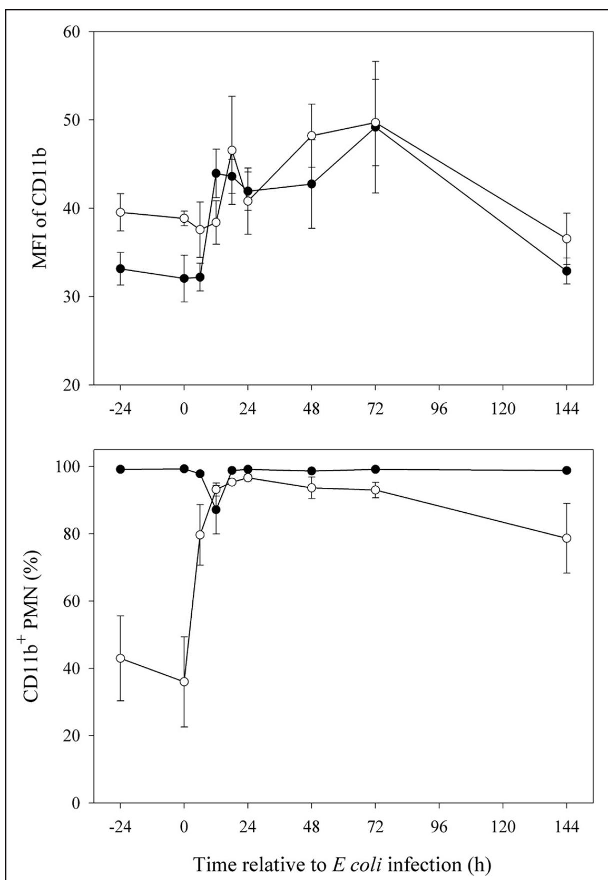

Figure 5—Mean (± SEM) values of MFI for CD11b on PMNs (top) and percentage of PMNs that were positive (+) for CD11b (bottom) in the blood (closed circles) and milk (open circles) versus time relative to *E coli*-induced mastitis in cows (n = 6).

Before inoculation, the percentage of milk $CD11b^+$ neutrophils was significantly (*P* = 0.005) lower (by 56%) than the percentage of $CD11b^+$ blood neutrophils (Figure 5). The percentage of blood $CD11b^+$ neutrophils decreased at 6 hours after *E coli* inoculation, whereas the percentage of milk $CD11b^+$ neutrophils increased. A significant (*P* < 0.001) decrease in the relative difference between blood $CD11b^+$ neutrophils and milk $CD11b^+$ neutrophils was detected at 6 hours, compared with control values. The significant (*P* < 0.001) convergence between the percentages of blood $CD11b^+$ neutrophils and milk $CD11b^+$ neutrophils persisted from 12 to 48 hours after *E coli* inoculation. The percentage of blood $CD11b^+$ neutrophils gradually returned to preinoculation values at approximately 24 hours after inoculation. However, the percentage of milk $CD11b^+$ neutrophils remained increased, compared with preinoculation values, until the end of the experimental period.

Discussion

In our study, we used flow cytometric analysis to investigate changes of CD62L and CD11b expression on neutrophils from blood samples and mammary secretions during *E coli*-induced mastitis. We found that milk neutrophils before inoculation had significantly lower CD62L expression and on average higher CD11b expression at their cell surface, compared with blood neutrophils. Differential expression of adhesion molecules at the neutrophil surface probably reflects the greater activation state of milk neutrophils, compared with blood neutrophils. This finding is consistent with previous results obtained with human, ovine, and bovine milk neutrophils.[21-24] However, the functional capacity of neutrophils seems to be compromised, presumably by their limited viability. Indeed, results of other studies[19,25] on bovine milk neutrophils, especially during the first month of lactation, indicate that milk neutrophils are functionally inferior to blood neutrophils. It may therefore be proposed that in our study, the low percentages of milk $CD62L^+$ and $CD11b^+$ neutrophils before *E coli* inoculation were related to the low viability of milk neutrophils. Senescent milk neutrophils probably undergo destructive processes that may lead to loss of protein integrity and therefore decreased antibody binding.

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Results of our study agree with those of previous reports[6-8] on bovine blood neutrophils, indicating that intramammary administration of *E coli* initiates a differential regulation of CD62L and CD11b expression. Findings in our study also indicate that expression of these adhesion molecules on milk neutrophils is oppositely modulated. The ability of blood neutrophils to effectively respond to inflammatory stimuli by dynamic recruitment of adhesion molecules has been proposed to play a pivotal role in the host defense against invading pathogens. This process requires a rapid disengagement of ligated CD62L molecules and upregulation of CD11b pools on neutrophils. Neonate cows and humans are highly susceptible to bacterial infections, and this has been associated with the relative inability of blood neutrophils to modulate CD11b expression.[26,27] Typical features of these 2 molecules following activation are the shedding of CD62L and the augmentation of CD11b expression on blood neutrophils.[4,5] The inverse modulation of the 2 molecules during coliform mastitis is possibly initiated by endotoxin released after death of the gram-negative bacteria in the mammary gland. Because endotoxin only sporadically reaches the circulation,[28] it has been proposed that the production of secondary inflammatory mediators, such as tumor necrosis factor-α, interleukin-1, interleukin-6, interleukin-8, complement factor 5a, and platelet-activating factor, plays a central role in the activation and recruitment of neutrophils during the acute-phase response.[5,29]

Milk SCCs increased at 6 hours after inoculation. Expression of CD62L at the surface of blood neutrophils was slightly decreased, compared with control values, whereas CD11b expression remained similar to control values. This observation might indicate that CD11b mobilization is not strictly necessary to induce the initial fast recruitment of neutrophils during mastitis caused by *E coli*. Indeed, early neutrophil adhesive responses can occur without prior mobilization of CD11b from internal stores,[30-32] and it is thought that basally expressed cell surface CD11b is converted to an active adhesive form upon cell stimulation.[33] However, the blood neutrophils examined in our study may not be representative of fully activated cells that are found during the initial inflammatory response. It has been recently proposed that the early events of the immune response during mastitis caused by *E coli* are initiated by means of a rapidly increased release of proinflammatory mediators mainly by epithelial cells.[34] Therefore, it might be argued that the marginating neutrophils were the primary contributors of the increase in milk SCCs for up to 6 hours after inoculation. Whether quantitative or qualitative changes in adhesion molecule expression had occurred on marginating neutrophils was not evaluated in our study.

During our entire study, CD62L expression was lower in milk neutrophils, compared with blood neutrophils. The decreased CD62L expression on milk neutrophils probably results from shedding of this molecule during the migratory process through the endothelium. Activation by *E coli* decreased the difference between the MFIs for CD62L on blood and milk neutrophils from 12 to 48 hours after *E coli* inoculation, reflecting the accelerated accumulation of neutrophils at the infection site. The greater epithelial permeability observed at this time may have facilitated neutrophil extravasation into the mammary gland, thereby lowering the differences between blood and milk neutrophils with respect to CD62L expression. Coinciding with the minimal MFI for CD62L on blood neutrophils observed between 12 and 18 hours after inoculation, blood neutrophil numbers decreased to the lowest values and fever peaked, indicating that the maximal inflammatory response was reached. In contrast, CD11b expression was upregulated to at least a similar extent in blood neutrophils, compared with milk neutrophils, from 12 to 48 hours after *E coli* inoculation. These results differ from that of a previous study[35] in which no detectable changes in CD11b expression on blood neutrophils of cows were found during *Staphylococcus aureus* infection. A possible explanation for this apparent discrepancy might reside in the different bacterial components, such as lipoteichoic acid and peptidoglycans for gram-positive bacteria and endotoxin for gram-negative bacteria. Indeed, it has been shown in mice that these immune activators elicit different responses in terms of leukocyte-endothelial cell interactions and leukocyte recruitment,[36] whereas on human monocytes, immune activators can differentially influence the expression of inflammatory surface receptors.[37] Nevertheless, findings similar to those of our study have been found in naturally infected cows with staphylococcal and streptococcal mastitis with respect to the amount of CD62L and β_2-integrin expression in blood neutrophils versus milk neutrophils.[23] In that study, however, milk neutrophils had a greater upregulation of CD18, compared with blood neutrophils. The higher milking rate, use of another pathogen, or both might account for this difference in our study.

On the basis of the intracellular amounts of CD11b in mature bovine blood neutrophils, a theoretic prediction of a maximum increase in surface expression of approximately 130% can be made.[38] However, the highest expression values in blood neutrophils measured in our study were only 37% or 55% above control values at 12 or 72 hours after inoculation, respectively. Therefore, it seems that only a small fraction of the intracellular pool may be necessary to fulfill the process of diapedesis during mastitis caused by *E coli*.

The decreased percentage of CD62L$^+$ and CD11b$^+$ blood neutrophils at 12 hours after inoculation, compared with preinoculation values, is possibly the result of the appearance of immature neutrophils as previously proposed.[39] Indeed, immature bovine neutrophils have been shown to express a lower percentage of CD11b, compared with their mature counterparts.[40] During the acute phase of inflammation, the percentage of CD62L$^+$ and CD11b$^+$ milk neutrophils increased until reaching similar values as detected for blood neutrophils. This increase in the percentage of CD62L$^+$ and CD11b$^+$ milk neutrophils seems to be related to the higher viability observed during the course of mastitis caused by *E coli*.

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Similar results have been obtained in previous studies[24,41] during mastitis elicited by *S aureus* (via, staphylococcal α-toxin) and *S epidermidis* infections.

[a]Provided by Bramley J, University of Vermont, Burlington, Vt.
[b]7-cm-long teat cannula, Medical Veterinary Materials, Deinze, Belgium.
[c]BD vacutainer, Plymouth, UK.
[d]Biokar Diagnostics, Beauvois, France.
[e]Ilyte, Instrumentation Laboratory, Zaventem, Belgium.
[f]Coulter Counter ZF, Coulter Electronics Ltd, Luton, UK.
[g]Fossomatic 360, Foss Electronic, Eden Prairie, Minn.
[h]Hemacolor, Merck KG&A, Darmstadt, Germany.
[i]Trizma base, Sigma Chemical Co, St Louis, Mo.
[j]Gibco Brl, Scotland, UK.
[k]Provided by Paape MJ, Immunology and Disease Resistance Laboratory, Agricultural Research Service, USDA, Beltsville, Md.
[l]ProBio, Margate Kent, UK.
[m]Dako A/S, Glostrup, Denmark.
[n]FACScan, Becton-Dickinson, San José, Calif.
[o]VMRD Inc, Pullman, Wash.

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