Effects of the Presence of the Mammary Gland on Expression of Neutrophil Adhesion Molecules and Myeloperoxidase Activity in Periparturient Dairy Cows

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ABSTRACT

Neutrophil function is reduced in periparturient dairy cows. Possible factors that reduce neutrophil function include endocrine changes associated with parturition and metabolic stresses associated with lactogenesis. In this study, mastectomized and intact cows were studied to specifically examine the effects of lactogenesis on neutrophil function in periparturient cows. Expression of adhesion molecules on neutrophils (L-selectin, mediating capture and rolling adhesion, and β2-integrin, mediating tight adhesion vital to egress) and neutrophil myeloperoxidase activity (an index of bactericidal activity) were assessed in mastectomized and intact cows. Expression of L-selectin decreased at parturition followed by rapid recovery to prepartum values in both intact and mastectomized cows. Expression of β2-integrins increased in intact cows at parturition but not in mastectomized cows. Expression of β2-integrins was greater in intact cows than in mastectomized cows throughout the study. Neutrophil myeloperoxidase activity decreased from baseline prepartum values as parturition approached in both intact and mastectomized cows. Expression of β2-integrins increased in intact cows at parturition but not in mastectomized cows. Expression of β2-integrins was greater in intact cows than in mastectomized cows throughout the study. Neutrophil myeloperoxidase activity decreased from baseline prepartum values as parturition approached in both intact and mastectomized cows, which suggests the endocrine changes associated with the act of parturition are predominant factors causing loss of neutrophil function. Myeloperoxidase activity recovered to prepartum values within a week of parturition in mastectomized cows; however, myeloperoxidase activity remained depressed in neutrophils obtained from intact cows throughout the first 20 d of lactation. The presence of the mammary gland and its attendant metabolic stresses slowed recovery of neutrophil function, which suggests that the metabolic stress of lactation exacerbated periparturient immunosuppression.

(Key words: periparturient immunosuppression, β2-integrin, L-selectin, myeloperoxidase)

Abbreviation key: ICAM = intercellular adhesion molecule, PAF = platelet-activating factor.

INTRODUCTION

Impaired neutrophil and lymphocyte function during the periparturient period is a contributing factor to the high incidence of infectious disease observed in the periparturient cow (6, 12, 13, 14, 16, 17). The diminished immune function not only increases susceptibility to new infections leading to such diseases as mastitis and metritis (23, 24, 31) but also can allow subclinical infection by microbes causing diseases such as salmonellosis and paratuberculosis to become clinical.

Neutrophils are part of the innate immune system; therefore, they play an important role as the first line of defense against infection. To be effective, neutrophils must be capable of receiving chemical signals that the body has been invaded by a microbe, and they must be able to egress from the blood stream at the site of microbial invasion. Neutrophils normally patrol endothelial surfaces, loosely binding to and rolling across the endothelium, ready to leave the vascular system wherever bacteria have invaded the body. This rolling adhesion along endothelial surfaces is mediated by the interaction of L-selectin, a protein on the surface of neutrophils, with carbohydrate ligands on the vascular endothelium (19, 26). When bacteria invade tissue, the endothelium of the postcapillary venules in that region up-regulate expression of several adhesion molecules, including an intercellular adhesion molecule (ICAM-1) in response to the inflammatory stimuli. The ICAM-1, in addition to the constitutively expressed ICAM-2 on the endothelial surface, will tightly bind proteins on the surface of neutrophils known as β2-integrins. This process allows neutrophils to become stationary long enough to migrate through the vascular endothelium. The expression of β2-integrins on neutrophils can be up-regulated in response to a variety of chemotactic factors, including complement fragment C5a, tumor necrosis factor, granulocyte-macrophage colony-stimulat-
ing factor, interleukin 8, and platelet-activating factor (PAF). These same stimuli tend to down-regulate expression of L-selectin on neutrophil surfaces (19, 26, 30).

We have previously demonstrated a dramatic decrease in the expression of L-selectin at parturition in Holstein cows followed by rapid recovery after parturition (21). Presumably, this down-regulation of L-selectin reduces the ability of neutrophils to detect and respond to a site of bacterial invasion. Expression of β2-integrins on neutrophils exhibited the opposite pattern, increasing gradually as parturition approached, peaking at calving, dropping immediately after calving, and then returning to initial values by 15 h postpartum both in resting and PAF-stimulated neutrophils (21).

Once neutrophils have successfully moved into the tissue at a site of invasion, they must migrate toward invading bacteria (chemotaxis), adhere to the bacteria, ingest the bacteria, and finally kill the bacteria. In vitro tests for all of these functions of neutrophils have been developed and, when applied to neutrophils of periparturient cows, most studies suggest marked impairment of chemotaxis and ability of neutrophils to generate bactericidal agents (6, 13, 16). Iodination of organic compounds catalyzed by neutrophil myeloperoxidase is one test used as an index of the ability of neutrophils to kill ingested bacteria. In previous studies, neutrophil myeloperoxidase activity was greatly diminished during the periparturient period (6, 13, 16). Greater suppression of myeloperoxidase activity was associated with susceptibility to infectious disease (6, 18).

What mediates the decline in neutrophil function associated with parturition and onset of lactation in the dairy cow? Many endocrine changes are associated with the act of parturition, including major increases in plasma concentrations of estrogen and cortisol, which may inhibit various aspects of the immune system. In addition, the onset of milk production imposes tremendous challenges to the mechanisms responsible for energy, protein, and mineral homeostasis in the cow (9, 10), which could be responsible for immunosuppression observed in periparturient dairy cattle. If the metabolic challenges associated with the onset of lactation are responsible for the decline in neutrophil function observed in the periparturient dairy cow, mastectomy should eliminate the decline in neutrophil function observed during the periparturient period. Mastectomy of pregnant dairy cows removes the impact of milk production while presumably maintaining endocrine as well as other changes associated with late pregnancy and parturition (K. Kimura and J. P. Gott, 1999, unpublished data). Neutrophils obtained from ten mastectomized and eight intact periparturient multiparous Jersey cows were evaluated to test this hypothesis.

**MATERIALS AND METHODS**

**Animals**

Eighteen multiparous Jersey cows between 5 and 8 yr of age were used in this study. Ten cows were mastectomized during the third to fifth month of gestation and were allowed to heal. The cows calved between February 1996 and January 1997. Eight intact cows, due to calve around the same time as each mastectomized cow, were chosen as controls so that reagents used on mastectomized cow neutrophils could also be used to assess neutrophil function of intact cows. This method reduced the effects that time and season might have had on the outcome of the experiment. Cows were managed as similarly as possible and cared for by the same caretakers for the duration of the study. Cattle were housed in a free-stall barn until 2 to 3 d before calving, when they were moved into maternity pens. Prior to and following calving, cows were fed alfalfa-corn silage based on a high dietary cation-anion difference [(Na+ + K+) – (Cl– + S2–)] = + 400 meq/kg. This type of diet is still fed on many dairy farms, and it is known to increase the probability of metabolic stress such as milk fever in cows.

Blood samples were collected by jugular venipuncture into tubes containing acid citrate dextrose for neutrophil assays. Samples were taken from 4 wk before the expected calving date through 4 wk after calving for adhesion molecule analysis and leukocyte count. Cows were bled twice a week for wk −4, −3, 3, and 4 around parturition, three times a week during wk −2 and 2, and daily during wk −1 and 1 around parturition. On the day of calving, samples were taken at 0, 12 and 24 h after calving. To assess neutrophil myeloperoxidase activity, blood samples were taken from six cows in each group, twice a week, from 3 wk prior to calving through 3 wk after calving. At each sampling time, blood samples from four non-pregnant heifers were also taken and used as internal laboratory controls for the myeloperoxidase assay, on the assumption that immune function in these animals was stable.

**Enumeration of Leukocytes and Leukograms**

Total leukocyte counts were determined in whole blood collected in tubes containing ethylenediaminetetraacetic acid using an electronic cell counter (CellTrack, Angel Engineering Corp., Trumbull, CT). The percentages of peripheral blood mononuclear cells and neutrophils were determined by flow cytometry (FACScan™; Becton Dickinson, San Jose, CA) using gating of these cells based on their forward and side scatter characteristics on dot plots (2). The number of neutrophils per
cubic millimeter of whole blood was calculated from these data.

L-Selectin and β2-Integrin Expression on Neutrophils

Monoclonal antibodies. Monoclonal antibodies against human L-selectin (0.1 mg/2 ml, Clone DREG-56, IgG1 Isotype; Pharmingen, San Diego, CA) and against the human β2 subunit of integrin (0.1 mg/ml, MHM23, LFA-1 β chain, IgG1 Isotype; DAKO Corp., Caprienteria, CA) were used. An isotype control antibody (0.1 mg/ml, Clone X-927, IgG1 Isotype; Pharmingen, San Diego, CA) and against human L-selectin (0.1 mg/ml, Clone DREG-56, IgG1 Isotype; Pharmingen, San Diego, CA) were used. An isotype control antibody (0.1 mg/ml, Clone X-927, IgG1 Isotype; DAKO Corp.) was also used to correct for nonspecific binding of the antibody to the cells. All antibodies were directly conjugated with fluorescein isothiocyanate (2).

Activation of neutrophils and immunostaining. Quantitation of adhesion molecules on the surface of neutrophils was done in the basal state and following stimulation with PAF. Fifty-microliter aliquots of whole blood were added to each of five wells in a microtiter plate (U-bottom, 96-well) allotted for each sample. Ten microliters of PAF (10 μg/ml) was added to two of the wells to activate neutrophils. The sample and PAF were incubated for 5 min at 39°C. The remaining three wells were treated with 10 μl of PBS only and remained in the basal, inactivated state. One PAF-treated well and one basal state well were treated with 20 μl of anti-L-selectin antibody. The other PAF-treated well and another basal state well were treated with 10 μl of anti-β2 integrin antibody. The remaining basal state well was treated with 10 μl of isotype control antibody. The plate was incubated with the monoclonal antibodies for 15 min at room temperature. Erythrocytes were then lysed with 150 μl of hypotonic lysing solution (2) for 90 s at room temperature. Next, 75 μl of hypertonic restoring solution (2) was added to restore the solution to isotonicity. The plate was centrifuged at 800 × g for 2 min, and the supernatant was discarded. This lysing process was repeated once more. The remaining leukocytes were washed once with 200 μl of PBS, and the cells were suspended in 200 μl of sheath fluid (Isoton II; Coulter Diagnostics, Hialeah, FL) for immediate flow cytometry analysis.

Flow cytometric analysis. A flow cytometer (FACScan™; Becton Dickinson, San Jose, CA) was used to acquire and analyze the neutrophil L-selectin and β2-integrin data. Data from 10,000 events per sample were acquired and analyzed using software (CellQuest™; Becton Dickinson, San Jose, CA). The neutrophil population was gated out from the other leukocyte populations based on their forward and side scatter characteristics on dot plots (2). The geometric mean fluorescent intensity of the fluorescein isothiocyanate in the histogram was directly related to the quantity of adhesion molecules on each cell.

Myeloperoxidase Assay

The ability of cells to incorporate 125I into trichloroacetic acid-precipitable material was determined as previously described (28). The assay was performed in cells stimulated with opsonized zymosan as well as in the absence of cells (to determine the extent of nonspecific adsorption). The total minus the nonspecific nanomoles of 125I incorporated/107 cells/h yielded the specific nanomoles of 125I incorporated/107 cells/h for each sample. Results from each animal were expressed as a percentage of the specific nanomoles of 125I incorporated/107 cells/h obtained in samples from four heifers (our internal laboratory standard) on each sampling day.

Statistical Analysis

For statistical purposes, data on adhesion molecule expression were grouped into weekly periods (wk –4 to 4) except around the time of calving (d –1, 0, 0.5, and 1). The weekly mean of individual animals was calculated from 2 (for wk –4, –3, 3, and 4), 3 (for wk –2 and 2) or 5 sampling times (for wk –1 and 1). For iodination, the data were arranged for d –20, –16, –12, –8, –4, 0, 4, 8, 12, 16, and 20. The data at each time point were the means of results obtained from one to three blood samples obtained during that time frame. To investigate the impact of mastectomy on relevant variables, we performed split-plot analyses of variance, followed by simple effect analyses in cases of significant (P < 0.05) interaction. The nonrepeated measure factor was treatment (intact vs. mastectomy), and the replication factor was cow. The repeated measure factor was time (d relative to parturition). Because of some imbalance in number of data points for cows, the ANOVA data were analyzed using the general linear model partial sums of squares procedure.

RESULTS

General Observations

Two mastectomized cows gave birth to twins. All intact cows developed milk fever within 24 h after calving, and they were treated intravenously with a calcium solution, from one to three times. Three intact cows also developed ketosis and displaced abomasum within 1 wk after calving. These cows were treated by intravenous glucose infusion and abomasopexy with a single suture inserted through the right ventral abdomen while being held in dorsal recumbency.
Enumeration of Leukocytes and Leukogram

The total white blood cell count increased prior to calving and decreased just after calving (time effect, $P = 0.0001$). This change was primarily attributable to an increase in neutrophils (time effect, $P = 0.0001$). Total white blood cell count was similar in intact and mastectomized cows throughout the periparturient period (Figures 1 and 2).

Flow Cytometric Analysis

Basal and PAF-stimulated neutrophil expressions of L-selectin are presented in Figures 3 and 4. Both mastectomized and intact cows exhibited a sudden decrease in expression of L-selectin at parturition, followed by an increase in L-selectin expression after parturition. Statistical analysis showed time ($P = 0.02$) and treatment (mastectomy) × time interaction ($P = 0.001$) effects but no treatment ($P = 0.977$) effect on basal state L-selectin expression. The PAF activation had no significant effect on L-selectin expression when compared with basal state L-selectin expression.
β2-integrin expression showed a completely different pattern (Figures 5 and 6). In intact cows, β2-integrin expression was up-regulated at parturition (from d –1 to d 1). Down-regulation followed parturition to a level below prepartum values. β2-integrin expression remained below prepartum values throughout the postpartum sampling period in intact cows. In mastectomized cows, β2-integrin expression was not up-regulated at parturition and tended to be lower than in intact cows throughout the sampling period. In vitro PAF stimulation up-regulated β2-integrin expression by 40 to 60%. β2-integrin expression following PAF stimulation exhibited the same pattern as constitutive expression, and there was no evidence of a difference in response to PAF between intact and mastectomized cows. Statistical analysis showed significant treatment (P = 0.04), time (P = 0.0001), and treatment × time interaction (P = 0.009) effects on basal state β2-integrin expression.

Myeloperoxidase Assay

In both mastectomized and intact cows, neutrophil myeloperoxidase activity decreased significantly from d -20 to calving in a similar manner (Figure 7). However, after calving, neutrophil myeloperoxidase activity of mastectomized cows began to increase so that by d 8 it had essentially returned to d -20 precalving levels. In contrast, neutrophil myeloperoxidase activity of intact cows remained low in all postcalving sampling periods. Statistical analysis showed significant effects of time (P = 0.001) and treatment × time interaction (P = 0.0004) effects but no treatment effect (P = 0.3).

DISCUSSION

Neutrophils play an important role in protection from infectious diseases such as mastitis (11, 29). Unfor-
imately, neutrophil function is greatly diminished in periparturient dairy cows (6, 13, 32), and the factors affecting neutrophil function are largely unknown. We found that elimination of milk production by mastectomy did not prevent the decline in neutrophil function at parturition (myeloperoxidase activity and down-regulation of L-selectin adhesion molecules), which has been observed in intact cows in this study and others (13, 21). The elimination of lactogenesis did allow rapid recovery of myeloperoxidase activity in neutrophils after calving but was not a factor in leukocyte cell count or L-selectin expression on neutrophils. Elimination of the mammary gland also reduced β2-integrin expression on neutrophils and prevented up-regulation of β2-integrin expression at parturition.

The neutrophil iodination reaction, which is utilized as an index of neutrophil bactericidal capability, is dependent upon a complex chain of events, which includes ingestion, oxidative metabolism, degranulation, and myeloperoxidase activity. Measurement of myeloperoxidase activity is generally accepted as a good test of neutrophil function (28). Impairment of neutrophil iodination is critical. Poor neutrophil myeloperoxidase activity has been associated with an increased incidence of infectious diseases of the mammary gland and reproductive tract in postpartum dairy cows (6, 7, 18), as well as with respiratory disease (27). In previous studies (6, 7, 13, 16), the myeloperoxidase activity of bovine neutrophils began to decrease approximately 2 wk prepartum, reached nadir during the first 7 to 10 d postpartum, and then recovered to prepartum values during the first 3 to 4 wk of lactation. In the present study similar results were observed up to parturition in both intact and mastectomized cows. However, following parturition, myeloperoxidase activity recovered quickly in mastectomized cows, whereas intact cows did not recover during the first 20 d of lactation (the length of the study). The stress associated with lactogenesis was particularly great in the intact cows because all intact cows developed milk fever. Several of these cows developed other metabolic diseases, suggesting that these intact cows were in profound negative calcium and energy balance states compared with the mastectomized animals. This finding was true, and the data are reported in a companion paper. An argument could be made that this study examined the effect of metabolic disease on neutrophil function rather than the effect of lactation on neutrophil function. Severe hypocalcemia (eliminated by mastectomy) seemed to be a plausible explanation for the prolonged reduction in neutrophil myeloperoxidase activity in the intact cows. However, in a previous study utilizing cows with and without milk fever (in which hypocalcemia was prevented by utilization of exogenous parathyroid hormone) (13), we observed no significant effect of milk fever on neutrophil function during the periparturient period. In this study the recovery of neutrophil myeloperoxidase activity in the mastectomized cows following parturition was faster than that reported in other studies, suggesting that milk synthesis and secretion significantly delay recovery of this function. We speculate that the negative energy balance associated with early lactation (10), eliminated by mastectomy (data presented in a companion paper), may have a major influence on recovery of neutrophil function following parturition.

The variation in the periparturient leukocyte cell count observed in both intact and mastectomized cows was attributable to transient changes in neutrophil number, in agreement with previous reports (8, 14, 15). The increase in neutrophils at parturition may be the result of decreased neutrophil rolling adhesion (margination) associated with down-regulated L-selectin expression. This down-regulation of L-selectin expression reduces the number of neutrophils adhering to endothelial surfaces, increasing the number of neutrophils free to circulate. This acute transient down-regulation of rolling adhesion reduces the number of the neutrophils patrolling the vasculature and could reduce egress of neutrophils into tissue in response to infection. Failure of neutrophils to enter tissues will increase the chance of infection (11, 29). L-selectin expression on neutrophils in intact and mastectomized periparturient cows was similar to the pattern previously observed. Our former study utilized intact cows that did not develop milk fever (21), which suggests that the act of parturition, rather than lactation and metabolic disease, decreased L-selectin expression.

β2-integrin expression in intact cows was similar to the pattern observed in our previous study with intact cows, which did not develop any metabolic diseases (21). In the previous study, β2-integrin expression after parturition was restored to normal levels within 3 d of parturition. However, in this study β2-integrin expression in intact cows remained lower than the preperturient value for an extended period following parturition. The failure to recover β2-integrin expression to the preperturient value in intact cows may reflect the influence of metabolic diseases, which developed after parturition in all intact cows.

β2-integrin expression was reduced by mastectomy in periparturient cows and did not increase at parturition as it did in intact cows. Thus, the dramatic change and greater β2-integrin expression during the sampling period in intact cows depended on the presence of the mammary gland (i.e., effect of lactogenesis and milk secretion). It is difficult to explain the significance of β2-integrin expression seen in intact cows. Higher levels of
expression do not necessarily mean higher adhesiveness because neither the constitutive nor the induced integrins are fully functional (4, 20, 25, 35) until stimulated by chemoattractants and divalent cations (1, 5). In contrast to β2-integrins, L-selectin is in a constitutively active state (3), although its affinity for ligands can be increased after stimuli (33).

The emigration of neutrophils from the vasculature is regulated by at least three distinct steps. The first step involves a capture and rolling adhesion mediated by the selectins. After this initial step, the leukocyte must be activated by chemoattractants, and the third step is firm adhesion or arrest of the cells to the endothelium by the activated integrins (22). An essential feature is that these steps act in sequence, not in parallel. The inhibition of any one of these steps gives complete, rather than partial, inhibition of neutrophil emigration (34). Therefore, down-regulation of L-selectin at parturition may render neutrophils unable to egress the vascular system despite up-regulation of β2-integrin expression at calving in intact cows. The acute decline in L-selectin expression observed in cows at parturition may be largely due to the effect of increased plasma cortisol concentrations, which occurs at parturition (2).

CONCLUSIONS

Neutrophil function and expression of the cell adhesion molecule L-selectin declined as parturition approached in both intact and mastectomized cows. These data suggested that removal of the mammary gland, which prevented lactogenesis and the attendant calcium, energy, and metabolic disease problems associated with lactogenesis, did not prevent a decline in neutrophil function in periparturient cows. This finding suggested that the act of parturition and its attendant endocrine changes were the likely causes of the decline in neutrophil function observed in both intact and mastectomized cows. Neutrophil myeloperoxidase activity recovered quickly following parturition in mastectomized cows but not in intact cows. These data suggested that lactogenesis and its associated disease problems delayed recovery of neutrophil function and contributed to the suppression of the immune system observed in the periparturient dairy cow. Future studies should attempt to identify the factors associated with parturition that are responsible for the initial decline in neutrophil function as well as factors associated with lactogenesis, which are responsible for the delay in neutrophil function recovery. This identification could allow intervention to prevent periparturient immune suppression.

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REFERENCES