Effects of Mastectomy on Composition of Peripheral Blood Mononuclear Cell Populations in Periparturient Dairy Cows

Kayoko Kimura, Jesse P. Goff, Marcus E. Kehrli, Jr., James A. Harp, and Brian J. Nonnecke
USDA, Agricultural Research Service,
National Animal Disease Center,
Periparturient Diseases of Cattle Research Unit,
Ames, IA 50010-0070

ABSTRACT

There is an increased incidence of infectious disease in periparturient dairy cows. During the periparturient period there is a decline in T-lymphocyte cell subsets, which parallels a reduction in functional capacities of blood lymphocytes and neutrophils. Mechanisms responsible for these changes in immune function during the periparturient period are poorly characterized. Ten mastectomized and eight intact multiparous Jersey cows were used to determine whether the periparturient changes in peripheral blood mononuclear cell populations are the result of the physiological demands associated with the onset of lactation or whether they are a result of the act of parturition. Blood mononuclear cells were phenotyped with monoclonal antibodies against T-cell subsets, B-cells, and monocytes. Blood samples were taken frequently from before 4 to 4 wk after parturition. In intact cows, all T-cell subset populations (i.e., CD3-, CD4-, CD8-, and gamma-delta positive cells) decreased at the time of parturition, while the percentage of monocytes increased. Mastectomy eliminated the changes in leukocyte subset populations (CD3-, CD4-, and gamma-delta positive cells, and monocytes) observed in intact cows around parturition. These results indicate that the mammary gland and metabolic stresses associated with lactation influence the composition of peripheral blood mononuclear cell populations in dairy cows during the periparturient period.

(Key words: immunosuppression, periparturient, peripheral blood mononuclear cell, T cell subset)

INTRODUCTION

Metabolic and infectious diseases in the periparturient dairy cow cause major economic losses due to decreased milk production and increased veterinary costs. Immune function is impaired during the periparturient period, contributing to the high incidence of infectious diseases (i.e., mastitis, Salmonellosis, Johne’s disease, etc.) observed in recently calved cows. We have previously demonstrated changes in the blood T-cell population, especially CD4-positive cells (T helper cells) and N12-positive cells (gamma-delta T cells) in periparturient cows (Kimura et al., 1999). These T-cell subset populations decline as parturition approaches, reach a nadir at calving, and recover several weeks after parturition, in about the same timeframe as the reduction in immune cell function observed in previous studies (Kashiwazaki, 1984; Kehrli and Goff, 1989; Kehrli et al., 1989; Cai et al., 1994).

Factors causing the decline in CD4- and N12-positive cell populations and immune cell function in periparturient cows are poorly characterized. Cows are subject to tremendous endocrine changes [increased levels of steroid hormones (estrogens, cortisol, etc.) and peptide hormones (growth hormone, prolactin)] associated with the act of parturition (Convey, 1974). At the same time, they are confronted by metabolic stressors (negative energy and calcium balance) associated with parturition and colostrogenesis (Grummer, 1993).

We hypothesized that the metabolic stressors associated with the onset of lactation contribute to the immunosuppression observed in periparturient cows. The intact and mammary gland and metabolic stresses associated with lactation (Goff et al., 2002). Ten mastectomized and eight intact multiparous Jersey cows were used in this study to determine whether compositional changes of peripheral blood mononuclear cells (PBMC) in periparturient cows result from the onset of lactation or endocrine changes and stresses associated with the act of parturition.

Received October 13, 2001.
Accepted January 15, 2002.
Corresponding author: J. P. Goff; e-mail: jgoff@nadc.ars.usda.gov.
1Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.
MATERIALS AND METHODS

Animals

Eighteen multiparous Jersey cows between 5 and 8 yr of age were evaluated in this study. Ten cows were mastectomized between 3 and 5 mo of gestation. A detailed description of the surgery can be found in the companion paper (Goff et al., 2002). Eight intact, age-matched cows calving around the same time as each mastectomized cow, served as controls. All cows calved between February 1996 and January 1997. Pregnant cows were housed in a free-stall barn. They were brought into maternity pens 2 to 3 d before calving. They were fed an alfalfa-corn grain based diet with a high dietary cation-anion difference [(Na⁺ + K⁺) - (Cl⁻ + SO₄⁻)] = +400 meq/kg] during the last 3 wk before parturition and for the first 4 wk of lactation (Goff et al., 2002).

Blood samples were taken by jugular venipuncture into tubes containing acid citrate dextrose (for phenotype analysis) or EDTA (for total leukocyte count) as anticoagulant. Samples were taken at regular intervals from 4 wk before the expected calving date to 4 wk after calving (at −27, −23, −19, −16, −13, −10, −8, −6, −5, −4, −3, −2, −1, 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, 19, 23, and 27 d around the time of parturition). Animal-related procedures were approved by the Animal Care and Use Committee of the National Animal Disease Center.

Cell Preparation and Antibody Binding for Analysis of Leukocyte Phenotype

Fifty microliters of whole blood with anticoagulant was incubated with 50 µl of primary mAb at room temperature for 15 min in a microtiter plate (U-bottom, 96-well) to allow binding of specific mAb (described below) with cell surface antigens. The cells were then treated with 150 µl of hypotonic lysis solution for 90 s at room temperature to lyse erythrocytes. Then, 75 µl of hypertonic restoring solution was added to restore the solution to isotonicity (Burton and Kehrli, 1995). The supernatant was decanted after centrifugation at 800 × g for 2 min. This lysing process was repeated once more. The leukocytes remaining were washed once with 200 µl of PBS and incubated at room temperature for 7.5 to 10 min with 50 µl of the secondary antibody described below. After incubation and one wash with PBS, the cells were suspended in 200 µl of sheath fluid (Isoton II, Coulter Diagnostics, Miami, FL) for immediate flow cytometric analysis.

Antibodies

The primary antibodies were all specifically reactive to bovine markers, which would allow segregation of PBMC into T cells, B cells, and monocytes. The T cells could be further subdivided into T-helper, T-cytotoxic/ suppressor, and gamma-delta T cells (Howard and Naessens, 1993; Ababou et al., 1994). Descriptions of working solutions of the primary antibodies are presented in Table 1. Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H+L) F(ab)’₂ (Caltag Laboratories, San Francisco, CA) or phycoerythrin-conjugated goat anti-mouse IgM (H+L) F(ab)’₂ (Southern Biotechnology Associates, Inc., Birmingham, AL) and were diluted 1:100 or 1:500, respectively, in PBS with 1% fetal bovine serum. In addition, the percentage of PBMC expressing IL-2r α chain and MHC Class II antigens was also assessed. Expression of IL-2r α chain is thought to be an index of the number of activated cells (Jackson et al., 1990). The MHC class II antigen is expressed constitutively on B cells and induced on monocytes during cell activation (Abbas et al., 1997).

Flow Cytometric Analysis

Leukocyte populations were characterized by flow cytometry as previously described (Kimura et al., 1999). Briefly, data from 5000 events/sample were acquired and PBMC populations were analyzed after gating out granulocyte populations.

Enumeration of PBMC

The total number of leukocytes was determined with an electronic cell counter (CellTrack, Angel Engineering Corp., Trumbull, CT). Differential cell counts were done using flow cytometry. Granulocytes and PBMC were identified based on forward and side-scatter characteristics on density plots (Burton and Kehrli, 1995). The total number of PBMC per cubic millimeter of whole blood was calculated from these data.

Statistical Analysis

Data were analyzed by split-plot repeated measure ANOVA, using the general linear model partial sums of squares procedure, followed by Duncan’s multiple comparisons. The statistical model included effects of treatment (intact versus mastectomy), time (days relative to parturition), and the interaction of treatment and time (treatment × time). The mean square error for the cow (treatment) was used as the error term to evaluate the effect of treatment. Residual error [time × cow (treatment)] was used to evaluate the effect of repeated measures factor, time, and its interaction with treatment (treatment × time). Differences were considered significant at P < 0.05.
### Results

#### General Observations

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

#### T Cell Populations

As parturition approached, intact cows exhibited a significant decline ($P < 0.05$) in the percentage of all T-lymphocyte cell subsets from d -27 levels, reaching a nadir around the time of calving (Figure 1). The CD3-positive cells (total T cells) decreased ($P < 0.05$) from $51.9 \pm 1.9\%$ (mean $\pm$ SEM) to $40.1 \pm 2.2\%$ and reached their nadir the day before calving. The CD4-positive cells, representing the T-helper cell population, decreased from $29.8 \pm 1.3\%$ to $24.8 \pm 2.3\%$ at calving ($P < 0.05$). The CD8-positive cells, representing the T-cytotoxic/suppressor cell population decreased from $12.3 \pm 2.4\%$ to $8.6 \pm 1.0\%$ just before calving ($P < 0.05$). The N12-positive cells, representing the gamma-delta T-cell population decreased from $7.2 \pm 1.0\%$ to $4.4 \pm 0.4\%$ at calving ($P < 0.05$). The T-lymphocyte cell populations slowly recovered after parturition, so that by d 27 after calving, T-cell population percentages were comparable to d-27 values, with the exception of the N12-positive cells, which did not recover completely. There were no significant reductions in T-cell subset populations in mastectomized cows during the periparturient period ($P > 0.05$). A significant treatment $\times$ time interaction as well (Table 2) in the percentages of CD3-, CD4-, and N12-positive cells indicated a significant difference between intact and mastectomized cows. Sustained low percentage of N12-positive cells in intact cows after parturition resulted in a significant treatment effect (Table 2).

#### CD4/CD8 ratio

The CD4 T-cell:CD8 T-cell ratios varied between 3.0 to 3.6 in intact cows and between 2.3 and 2.9 in mastectomized cows with no definite pattern during the sampling period. The difference between treatments was not significant (Table 2).

#### Interleukin-2 Receptor (IL-2r)

The percentage of PBMC expressing the IL-2r $\alpha$ chain (an index of “activated” cells) varied from day to day in both groups (Figure 2), and there were no significant treatment, time, or treatment $\times$ time interaction effects (Table 2). There was a trend toward decreased IL-2r positive cells in intact cows from d -8 to d 0.5 ($0.05 < P < 0.1$), with the nadir occurring on d 0.

#### Major Histocompatibility Complex (MHC) Class II Antigen Expression

There was a significant difference in the percentage of cells expressing MHC class II antigen between intact and mastectomized cows at the start of the sampling period. On d -27, the percentage of cells positive to MHC class II in intact cows ($32.4 \pm 2.0\%$) exceeded the percentage in mastectomized cows ($25.9 \pm 2.2\%$). In intact cows, the percentage of cells positive to MHC class II decreased at parturition and remained low after calving (Figure 2). Mastectomized cows exhibited no

---

**Table 1.** Primary antibodies used to identify mononuclear cells.1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>mAb clone (murine)</th>
<th>Isotype</th>
<th>Working mAb concentration (µg/ml)2</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>MM1A</td>
<td>IgG1</td>
<td>7</td>
<td>Total-T lymphocyte</td>
</tr>
<tr>
<td>CD4</td>
<td>GC50A1</td>
<td>IgM</td>
<td>14</td>
<td>T-helper lymphocyte</td>
</tr>
<tr>
<td>CD8</td>
<td>CACT80C</td>
<td>IgG1</td>
<td>21</td>
<td>T-cytotoxic lymphocyte</td>
</tr>
<tr>
<td>N12</td>
<td>CACT61A</td>
<td>IgM</td>
<td>14</td>
<td>$\gamma\delta$ T lymphocyte receptor</td>
</tr>
<tr>
<td>IL-2r</td>
<td>CACT108A</td>
<td>IgG2</td>
<td>28</td>
<td>Interleukin-2 receptor $\alpha$ chain</td>
</tr>
<tr>
<td>MHC2 class II</td>
<td>TH14B</td>
<td>IgG2</td>
<td>3.5</td>
<td>Class II major histocompatibility complex</td>
</tr>
<tr>
<td>B Lymphocyte</td>
<td>BAQ155A</td>
<td>IgG1</td>
<td>3.5</td>
<td>B Lymphocyte</td>
</tr>
<tr>
<td>Monocyte</td>
<td>BAQ151A</td>
<td>IgG1</td>
<td>3.5</td>
<td>Monocyte</td>
</tr>
</tbody>
</table>

1The source of all mAb was VMRD Inc. (Pullman, WA), and the original concentration of the mAb solution was 1 mg/ml.

2mAb diluted in PBS with 1% fetal bovine serum.

3MHC = Major histocompatibility complex.
significant decline in cells positive to MHC class II during the sampling period. Although there was a significant decline in cells positive to MHC class II after parturition in intact cows, the values were still higher than in mastectomized cows.

**B Cells and Monocytes**

The B-cell percentages showed no significant changes during the sampling period in either group (Figure 3, Table 2). There was a significant increase \( (P < 0.05) \) in the percentage of monocytes in intact cows with the approach of parturition and a significant decrease \( (P < 0.05) \) after parturition with the zenith occurring at calving \( (29.0 \pm 2.4\%); \) Figure 3). In mastectomized cows, the percentage of monocytes declined slightly but not significantly as parturition approached. The difference in monocyte percentages between treatment groups was significant (Table 2).

**Enumeration of Leukocytes**

In both intact and mastectomized cows, there was a significant increase \( (P < 0.05) \) in total leukocytes before calving and a significant decrease \( (P < 0.05) \) after parturition (Figure 4). Time was the only significant factor affecting the number of circulating leukocytes (Table 2). The total number of PBMC decreased slightly at parturition compared with d -17 levels in both groups;

---

**Figure 1.** Percentage of total T-lymphocytes (a), T-helper lymphocytes (b), T-cytotoxic/suppressor cell (c), and gamma-delta-T lymphocytes (d) in peripheral whole blood from intact (n = 8; □) and mastectomized cows (n = 10; □) during the periparturient period.
Table 2. The results (P values) of analysis of variance for leukocyte phenotype (% of total blood leukocytes) and leukocyte count (cells/mm$^3$).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Treatment</th>
<th>Time</th>
<th>Treatment × time</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD4</td>
<td>NS$^5$</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD8</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>N12$^1$</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-2r$^2$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MHC$^3$class II</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>B cell</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Monocyte</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total leukocytes count</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>PBMC$^4$ count</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^1$ N12 = Gamma-delta T cell receptor.
$^2$ IL-2r = Interleukin-2 receptor.
$^3$ MHC = Major histocompatibility complex.
$^4$ PBMC = Peripheral blood mononuclear cells.
$^5$ NS = Not significant.

however, there was no overall time effect in either group (Figure 4, Table 2).

**DISCUSSION**

In intact cows, the percentage of T-lymphocytes and T-cell subsets comprising the PBMC declined significantly as parturition approached, reaching a nadir at parturition. Changes were most evident from -2 to 2 wk around parturition. The MHC class II antigen is thought to be expressed constitutively on B cells and induced on monocytes during cell activation. In intact cows, there was no significant decrease in B cells and a significant increase in monocytes at calving. However, the percentage of PBMC expressing MHC class II antigens actually declined at calving. This suggests that the percentage of activated monocytes had actually fallen and reached a nadir at calving.

The observation that there was a higher percentage of cells positive to the MHC-II antigens in intact cows than in mastectomized cows in later gestation deserves further attention. The work of Van Kampen and Mallard (1997) demonstrated that cells positive to MHC-II in nonpregnant, nonlactating cows comprised about 48% of PBMC. Eight weeks before calving at the end of the previous lactation, cells positive to MHC-II comprised just 39% of PBMC. Three weeks before calving and at calving, percentages of cells positive to MHC-II were similar to that of nonpregnant, nonlactating cows. Together with our data, the presence of the mammary gland in late pregnancy is somehow stimulating expression of the MHC-II antigen in late gestation. One factor known to upregulate MHC-II expression is tumor necrosis factor-alpha (Rimstad et al., 1995). Concentrations of tumor necrosis factor-alpha are very high in mammary secretions during the dry period (Rewinski and Yang, 1994). Elimination of this source of tumor necrosis factor-alpha by mastectomy may have prevented the upregulation of the MHC-II antigen normally observed in late gestation.

The changes in immune cell populations observed in the intact periparturient cows of this study are very similar to those previously reported in our laboratory (Kimura et al., 1999). Shafer-Weaver et al., (1996) demonstrated an association between decreased T-cell subsets [CD2-, CD4-, CD8-, and CD5- (an antigen expressed by all T cells and a subset of B cells) positive cells] and increased monocyte populations in periparturient cows and diminished lymphocyte function when compared to nonlactating cows.

Journal of Dairy Science Vol. 85, No. 6, 2002
Mastectomy eliminated almost all the changes in leukocyte subset populations seen in intact cows around the time of parturition. Because there were no significant changes in PBMC population, percentages in mastectomized cows, we can conclude that the act of parturition, despite its attendant changes in feed intake, plasma estrogens and cortisol (see companion paper, Goff et al., 2002), is not the main factor responsible for the immune cell population changes observed in the intact cows. Presumably, then, the changes in PBMC populations are the result of the onset of lactation. How does the presence of the mammary gland affect immune function?

One possibility is that the mammary gland is selectively removing T cells from the blood and sequestering them within the mammary gland. Blood flow and migration of leukocytes into the mammary gland does increase as the gland prepares for milk production (Gorwit et al., 1989; Metcalf et al., 1992). However, colostrum leukocytes consist predominantly of monocytes, and to a lesser extent lymphocytes and neutrophils (Nickerson, 1989). Also, Shafer-Weaver et al. (1996) showed that the changes in mammary gland mononuclear cell populations reflected similar changes in PBMC populations. In addition, Park et al. (1992) found that mammary gland secretions from cows within 48 h after parturition had fewer CD2- (expressed on all T cells except gamma-delta T cells), CD4-, and CD8-positive cells than secretions from lactating or nonlactating gland. Taylor et al. (1994) also found fewer CD4-positive cells in mammary gland secretions obtained in early lactation. Based on these studies, changes in PBMC populations observed in the present study were not likely due to selective sequestration of particular T-lymphocyte populations within the mammary gland.

The changes in PBMC subset populations may reflect changes in the metabolic state of the cows induced by...
the onset of lactation. As reported in the companion paper (Goff et al., 2002), mastectomy eliminated the hypocalcemia observed in intact cows and greatly improved energy balance (utilizing plasma NEFA concentration as an index of body fat mobilization) during the periparturient period (Goff et al., 2002). Because all the intact cows developed milk fever and three developed ketosis, it is tempting to theorize that changes in immune cell population observed in the intact cows were secondary to these diseases. However, it has been difficult to demonstrate that the periparturient impairment of immune function or changes in PBMC populations in cows with milk fever were significantly greater than in cows without milk fever (Kehrli and Goff, 1989; Kimura et al., 1999). Other researchers also found there was no effect of metabolic disease (ketosis or milk fever) on the population of any subset of PBMC in periparturient dairy cows (Van Kampen and Mallard, 1997). Development of milk fever does not appear to exacerbate immunosuppression observed in periparturient dairy cows (Kehrli and Goff, 1989). This remains somewhat perplexing because cows developing milk fever or ketosis are at increased risk of developing mastitis and metritis (Curtis et al., 1983; Correa et al., 1993). It seems clear from this study that the presence of the mammary gland is the predominant cause of the immune cell population changes observed in the periparturient cows. Is an immunosuppressive compound elicited by the mammary gland in late gestation and in just the first weeks of lactation? Or is the negative calcium and energy balance (or perhaps the fluctuating alpha-tocopherol, beta-carotene, and retinoic acid concentrations) reported in the periparturient cow responsible for the immune cell population changes? Because milk fever and ketosis are simply more severe cases of preexisting conditions (negative calcium or energy balance) it may be impossible for us to discern differences between the effects of severe and moderate imbalances on immune cell parameters, especially when examining immune parameters in limited numbers of animals.

Other possibilities include increased death of T lymphocytes or redistribution of T lymphocytes to other tissues such as lymphoid organs. Both events are known to occur after administration of glucocorticoids (Dhabhar et al., 1995; Burton and Kehrli, 1996; Cidlowksi et al., 1996). However, changes in the PBMC populations observed in our study are difficult to attribute to the acute rise in cortisol observed in cows at parturition, because changes in the PBMC occurred before the increase in plasma cortisol. Furthermore, there were no significant differences in cortisol concentrations between intact and mastectomized cows. In the plasma of mastectomized cows, the concentrations of other steroid hormones that might affect T-lymphocyte apoptosis or redistribution were similar (progesterone) or higher (estrone and estradiol) than in intact cows (Goff et al., 2002).

PBMC are composed of T and B lymphocytes and monocytes. We have suggested that T-lymphocyte numbers are declining because the percentage of T lymphocytes declines at parturition. However, there is also a rise in the percentage of monocytes at calving. One could also interpret the data to mean that the decline in the percentage of T lymphocytes is simply a consequence of the increase in the percentage of monocytes and should not be interpreted as an index of immune suppression. The epidemiological evidence of increased disease in the periparturient period more strongly supports the interpretation that the declining percentage of T-lymphocytes, essential to cell-mediated immunity, is playing a role in periparturient immune suppression.

**CONCLUSION**

In intact cows, there is a decline in the percentage of T lymphocytes within the PBMC as parturition approaches. These population changes have previously been shown to be associated with the immune suppression commonly observed in periparturient cows. Mastectomy eliminated many of these changes, especially those associated with T-cell subsets and monocytes; thus, the act of parturition with its attendant effects on feed intake and steroid and peptide hormone profiles is not the major immunosuppressive factor in the periparturient cow. These results suggest that the presence of the mammary gland is responsible for most of the changes in the composition of PBMC populations observed in periparturient dairy cows. These results suggest: 1) the mammary gland may produce substances that directly affect immune cell populations, or 2) metabolic demands associated with the onset of lactation negatively impact the composition of circulating PBMC populations.

**ACKNOWLEDGMENTS**

The authors thank Norman S. Tjelmeland, Creig E. Caruth, Eugene C. Rieks for animal care and Robert F. Strahan for advice regarding statistical analyses. Expert surgical assistance was provided by Syd Hartman and Roger Spaete.

**REFERENCES**


Nickerson, S. C. 1989. Immunological aspects of mammary involu-


