Effects of Preventing Periparturient Hypocalcemia in Cows by Parathyroid Hormone Administration on Hematology, Conglutinin, Immunoglobulin, and Shedding of Staphylococcus aureus in Milk

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ABSTRACT

The effects of hypocalcemia at parturition on concentrations of serum immunoglobulin and conglutinin, number of bacteria shed into milk, and leukograms of dairy cows were investigated from -4 wk prepartum to 4 wk postpartum. Ten healthy multiparous Holstein cows were fed a high calcium diet to induce hypocalcemia at parturition. Five cows received intramuscular parathyroid hormone to prevent hypocalcemia at parturition. All cows experienced a leukopenia (attributable to an absolute and relative neutropenia) during the 1st wk after calving, decreased serum conglutinin activity during the first 3 wk postpartum, and decreased concentration of serum IgG1 during the 3 wk before calving. At parturition, a large increase in organisms was found in foremilk (1000 to 10,000 times more than prepartum values). Neither the hematological changes nor the decreased immunoglobulin concentration was influenced by hypocalcemia or the development of milk fever. This implies that the degree of hypocalcemia observed did not have a large or irreversible influence on bacterial infection, hematological, or humoral immunity changes in periparturient cows.

INTRODUCTION

The bovine mammary gland is most susceptible to infection and clinical disease during the periparturient period (18, 20, 25). During this period, adverse changes in immune cell function occur that increase the susceptibility of the bovine mammary gland to infection and clinical mastitis (12, 13, 14, 15, 16, 17, 19, 28). Although a causal relationship has not been established, cows with parturient hypocalcemia (PH) have a five to eight times greater chance of mastitis and a nine times greater chance of having coliform mastitis than if PH is absent (5).

Parturient hypocalcemia is primarily a disorder of calcium homeostasis, associated with the onset of lactation in dairy cows. Plasma concentrations of the hormones 1,25-dihydroxyvitamin D [1,25-(OH)2D] and parathyroid hormone (PTH), which regulate calcium homeostasis, increase greatly at parturition in the bovine animal. The paracrine role of 1,25-(OH)2D in local immune responses and the possible effects of changes in plasma 1,25-(OH)2D concentrations at parturition in the dairy cow have been reviewed (23).

The objective of the present study was to contrast the effects of normocalcemia (attained by PTH treatment) and hypocalcemia on the leukogram, and serum concentrations of immunoglobulin, complement, and conglutinin during the periparturient period.

Received October 10, 1989.
Accepted February 5, 1990.
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1990 J Dairy Sci 73:2103-2111 2103
MATERIALS AND METHODS

Animals and Experimental Design

Ten healthy multiparous Holstein cows were used during the periparturient period from 4 wk prepartum to 4 wk postpartum. All gestation periods were synchronized to minimize the duration of the study. As described in a previous report (7), all cows received a high calcium diet during the prepartum period so that they would be predisposed to PH and milk fever after calving. Cows were randomly assigned to two experimental groups. One group of five cows was given crude synthetic PTH (1-34 N-terminal fragment, Peninsula Laboratories, Inc., San Carlos, CA) beginning approximately 7 d (average of 6.4 d) prepartum with an intramuscular 20-mg dose every 8 h for the first six treatments as a loading dose (6). Maintenance doses of 10 mg of PTH were continued every 8 h until parturition, at which time 20 mg of PTH were administered three times at 8-h intervals. Cows were then gradually withdrawn from PTH therapy by 50% reductions in dose per day down to 2.5 mg/8 h. Withdrawal from PTH was completed after 48 h of treatment at 2.5 mg/8 h (average, by 6.25 d postpartum). Five control cows were treated with the carrier used in the PTH preparation following the aforementioned time schedule.

Blood samples were obtained once a week beginning approximately 4 wk before the expected calving date. The frequency of sampling was increased to a Monday, Wednesday, Friday schedule about 2 wk before expected parturition and continued at that frequency for at least 2 wk postpartum. For the final 2 wk, blood samples were obtained from cows once a week.

Leukogram Determinations

Blood was collected by jugular venipuncture into EDTA-containing tubes. Total leukocyte counts were determined by electronic counting (CellTrack, Angel Engineering, Corp, Trumbull, CT). Slides for differential cell counting were prepared by cytocentrifugation, stained with a combination Wright's/Giemsa stain, and 200 cells were differentiated into neutrophils, eosinophils, or mononuclear cells (since this method cannot accurately discern lymphocytes from monocytes in bovine blood). Relative proportions and total number of each cell population/μL of blood were calculated.

Serum Immunoglobulin Isotypes, Complement, and Conglutinin Activity

Serum was collected from jugular venipuncture blood samples and was frozen at -70°C until tested. Immunoglobulin isotypes (IgM, IgG1, IgG2) were determined by radial immunodiffusion using commercially available kits (VET-RID, Bethyl Labs, Inc., Montgomery, TX). Complement activity was determined by two methods. The first method measured the diameter of hemolysis induced by bovine sera in agar gel containing guinea pig erythrocytes opsonized with specific bovine antisera (26). In the second method, bacteriostatic activity associated with serum complement was determined with a serum-sensitive strain (Eng) of Escherichia coli. Serum conglutinin activity was determined by a complement-dependent agglutination of E. coli (27).

Specific Antibody Titers

α- Staphylococcus aureus titers. An enzyme-linked immunosorbent assay was used as described by Opdebeeck and Norcross (21). Briefly, the antigen used was S. aureus (strain 2-8) exopolysaccharide coated onto microtiter plates by adding 1 ml/well for 2 h at 37°C and incubating overnight at 4°C. Plates were washed five times with phosphate-buffered saline (PBS, with Tween 20). Unknown samples of serum were diluted 1:50 in PBS and added to wells for 2 h at 37°C. Wells were washed again with PBS, and peroxidase conjugated goat α-bovine immunoglobulin was added for 40 min at 37°C. Wells were washed again with PBS, and peroxidase conjugated goat α-bovine immunoglobulin was added for 40 min at 37°C. Wells were washed again, and substrate (2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) was added for 5 min with shaking. Optical density at 405 nm of each well was determined and recorded with a spectrophotometer plate reader.

α-Cryptosporidium parvum titers. An enzyme-linked immunosorbent assay was used as described by Harp et al. (8). Briefly, microtitation plates (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with antigen by incubating with disrupted C. parvum oocysts
overnight at 4°C. Before assays were run, plates were blocked by incubation with 1% fish skin gelatin (Norland Products, Inc., New Brunswick, NJ) in PBS for 2 h at 37°C. Plates were rinsed four times with PBS containing 2.5% nonfat dry milk, .01% antifoam A (Sigma Chemical Co., St. Louis, MO), and .05% Tween 20 (Sigma Chemical Co., St. Louis, MO). Twofold dilutions of serum were added and were incubated for 1 h at 37°C. After rinsing, rabbit α-bovine Ig (Oako Corp., Santa Barbara, CA) was added to each well, and plates were incubated 1 h at 37°C. Plates were rinsed, and goat α-rabbit Ig-alkaline phosphatase conjugate (Accurate Chemical, Westbury, NY) was added. Plates were incubated 1 h at 37°C and rinsed, and para-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added to each well. Plates were incubated in the dark for 1 h at 22°C, followed by overnight incubation at 4°C, and read with an automated plate reader set on dual wavelengths of 405 and 610 nm.

Bacterial Shedding from Infected Mammary Glands

Cows were experimentally infected by intracisternal administration of ~50 cfu of S. aureus (Newbould strain 305, ATCC 29740) in one or two mammary quarters at least 6 mo prior to this study. These intramammary infections (IMI) persisted in one cow per treatment group throughout the study.

Lacteal secretions from infected quarters were collected at 2- to 4-d intervals beginning 1 wk prepartum and ending ~6 wk postpartum for quantitative bacterial analysis. Decimal dilutions of sonicated milk samples were plated onto sheep blood agar plates, incubated at 39°C, and examined at 24 and 48 h for numbers of bacterial colonies. Milk was sonicated (1 W for 10 s) to break up clusters of staphylococci and disrupt phagocytes containing viable bacteria.

Data Analysis

Data from each cow were coded relative to actual calving dates. Values for individual cows were averaged within each week relative to calving. To compare the effect of PTH administration, data from 2 wk prepartum were chosen as the pretreatment baseline values for each parameter. Changes for each cow from baseline were determined and averaged by treatment group for each of the following 3 wk. Differences (P<.05) between the average changes of PTH-treated cows and controls were determined by Student’s t test.

To evaluate changes across treatments for each cow, individual cow values within each week relative to the day of calving (d 0) were averaged and analyzed by fitting the general linear model: y = mean + week + cow + error. In this model, the data were blocked by week (a 7-d period before or after the time of calving) and by cow (representing animal differences), and error represented the residual animal variation after fitting the above model. Significant differences between wk-2 and each of the 3 successive wk were judged by F tests of the week effect. Probabilities were considered significant if P<.05.

RESULTS

General Observations at Parturition

One control cow developed parturient paresis and required treatment with intravenous calcium borogluconate solution; another cow had difficulty standing but treatment with calcium was not necessary. All controls had retained placentas 12 h after calving, and developed metritis and clinical illness. Two cows receiving PTH gave birth to twins and had retained placentas. One cow receiving PTH became weak and febrile after calving, was treated symptomatically, and ultimately euthanatized 2 d postpartum. No renal or hepatic damage was evident on postmortem and no diagnosis for the cause of clinical illness was obtained (7). Data obtained from this cow prior to her clinical illness were retained because the results were not disparate from other cows.

Intramammary Infection Status

The two cows (one treated with PTH and one control) with persistent S. aureus IMI exhibited clinical mastitis (atypical secretions, fever, and inappetence) and had more than 10⁷ cfu of S. aureus/ml of milk within 2 d after...
Figure 1. Shedding of *Staphylococcus aureus* from mammary secretions of two Holstein cows during the peripartal period. Data are the mean log\(_{10}\) number of colony-forming units of bacteria per milliliter of secretion. PTH = Parathyroid hormone.

Figure 2. Effect of intramuscular parathyroid hormone (PTH) administration on leukograms of periparturient cows compared with placebo-injected control cows. Data are the total number of cells/µl of blood ± SEM (n = five cows/group).

Perlparturient Plasma Calcium and 1,25-Hydroxyvitamin D Concentrations

Plasma calcium concentrations were reported previously (7). In brief, plasma calcium concentrations in the nontreated controls (milk fever prone) decreased within 24 h after parturition to below 7 mg/100 ml. Blood calcium of cows receiving exogenous PTH remained above 9 mg/100 ml. Blood concentrations of 1,25-(OH)\(_2\)D increased in both groups of cows, peaking approximately 7 d earlier in the cows receiving exogenous PTH (7).
Changes in Leukogram

Leukograms from nontreated controls and PTH-treated cows were similar throughout the periparturient period despite differences in plasma calcium concentration around the time of parturition (Figure 2). Data from both treatment groups were analyzed as one group to determine significant changes in leukograms attributable to calving (Table 1). All cows were leukopenic during the 1st wk after calving (a 30% drop in total circulating leukocytes). This leukopenia was primarily due to a neutropenia (57% absolute decline in the number of circulating neutrophils). Although no significant changes in the number of circulating mononuclear cells throughout the immediate periparturient period were observed, a slight but significant increase was found in circulating mononuclear cells during the 3rd and 4th wk after calving.

Changes in Serum Immunoglobulin Concentrations

Differences in total serum IgM or IgG1 concentrations were not observed between PTH-treated cows and untreated controls, during or after PTH administration (Figures 3a and 3b). Administration of PTH was associated with an increase in total serum IgG2 as shown in Figure 3c. Differences between the two groups of cows in antibody titers to S. aureus or C. parvum were not detected (Figure 4a and 4b).

Data from both groups were analyzed as one group to determine changes in serum immunoglobulin concentrations attributable to calving. Significant decreases in serum IgG1 concentrations were detected during the periparturient period in all cows (Table 1). Serum concentrations of other isotypes of immunoglobulin did not decrease during the periparturient period.

Changes in Serum Complement and Conglutinin Concentration

Changes in serum complement activity were not detected by either the HIG or bacteriostasis assays (Table 1). No difference in serum conglutination activity between the PTH-treated cows and the placebo-injected control cows was observed (Figure 5). However, the conglutinating activity of serum from all 10 periparturient cows for E. coli declined during the periparturient period. Mean reciprocal log2 conglutinin titers initially ranged from 6.2 to 7.0 for the immediate 3 wk prepartum, dropped...
TABLE 1. Weekly averages for various cell counts in blood, serum immunoglobulin, conglutinin titers, hemolytic complement (hemolysis in gel), and bacteriostasis activity measured during the periparturient period of 10 multiparous Holstein cows. Weeks were calculated as 168 h periods before or after the hour of calving. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Week relative to parturition</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Total leukocytes/µl</td>
<td>7000 ± 300</td>
<td>7300 ± 300</td>
<td>7900 ± 500</td>
<td>5500 ± 500</td>
<td>6500 ± 500</td>
<td>7500 ± 500</td>
<td>8000 ± 1000</td>
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<td>Neutrophil count/µl</td>
<td>2220 ± 260</td>
<td>2700 ± 240</td>
<td>3500 ± 400</td>
<td>1500 ± 400</td>
<td>2000 ± 400</td>
<td>2200 ± 400</td>
<td>2000 ± 400</td>
</tr>
<tr>
<td>Neutrophil relative %</td>
<td>31 ± 3.7</td>
<td>37 ± 3.2</td>
<td>42 ± 3.7</td>
<td>24 ± 3.7</td>
<td>29 ± 4.0</td>
<td>27 ± 4.0</td>
<td>24 ± 3.7</td>
</tr>
<tr>
<td>Eosinophil count/µl</td>
<td>4 ± 50.0</td>
<td>200 ± 50.0</td>
<td>107 ± 20.0</td>
<td>45 ± 14.0</td>
<td>29 ± 7.0</td>
<td>180 ± 40.0</td>
<td>570 ± 180.0</td>
</tr>
<tr>
<td>Eosinophil relative %</td>
<td>63 ± 3</td>
<td>58 ± 3</td>
<td>53 ± 3</td>
<td>73 ± 4</td>
<td>67 ± 4</td>
<td>69 ± 4</td>
<td>68 ± 3</td>
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<tr>
<td>Mononuclear cell count/µl</td>
<td>63 ± 2.5</td>
<td>58 ± 3</td>
<td>53 ± 3</td>
<td>73 ± 4</td>
<td>67 ± 4</td>
<td>69 ± 4</td>
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<td>Mononuclear cell relative %</td>
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<tr>
<td>IgM1</td>
<td>297 ± 24.0</td>
<td>274 ± 24.0</td>
<td>390 ± 19.0</td>
<td>262 ± 30.0</td>
<td>201 ± 15.0</td>
<td>190 ± 16.0</td>
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<td>IgG1</td>
<td>777 ± 64.0</td>
<td>489 ± 86.0</td>
<td>273 ± 40.0</td>
<td>447 ± 43.0</td>
<td>848 ± 59.0</td>
<td>981 ± 76.0</td>
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<tr>
<td>IgG2</td>
<td>2000 ± 173.0</td>
<td>2044 ± 169.0</td>
<td>2196 ± 217.0</td>
<td>2133 ± 236.0</td>
<td>2265 ± 228.0</td>
<td>2566 ± 250.0</td>
<td>2656 ± 252.0</td>
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<tr>
<td>Conglutinin activity2</td>
<td>6.90 ± 0.07</td>
<td>6.68 ± 0.2</td>
<td>6.23 ± 0.16</td>
<td>4.0 ± 0.5</td>
<td>3.3 ± 0.4</td>
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<td>5.1 ± 0.6</td>
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<tr>
<td>Hemolysis in gel3</td>
<td>109 ± 3</td>
<td>111 ± 3</td>
<td>115 ± 3</td>
<td>111 ± 4</td>
<td>118 ± 4</td>
<td>121 ± 3</td>
<td>129 ± 3</td>
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<tr>
<td>Bacteriostasis4</td>
<td>43 ± 4</td>
<td>36 ± 4</td>
<td>41 ± 3</td>
<td>40 ± 5</td>
<td>43 ± 4</td>
<td>40 ± 4</td>
<td>46 ± 6</td>
</tr>
</tbody>
</table>

1 Milligrams per deciliter of serum. Data are presented as the mean ± SEM.
2 Reciprocal log2 titer.
3 Percent of an internal assay standard sera diameter of hemolysis.
4 Percentage transmittance at 540 nm.
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Figure 4. Effect of intramuscular parathyroid hormone (PTH) administration in periparturient cows compared with placebo-injected control cows on specific a) anti-Staphylococcus aureus antibody (data are optical density readings at 405 nm ± SEM determined by enzyme-linked immunosorbant assay. n = five cows/group), and b) anti-Cryptosporidium parvum antibody (data are mean reciprocal titer log2 ± SEM determined by ELISA. n = four cows/group).

Figure 5. Effect of intramuscular parathyroid hormone (PTH) administration on serum conglutinin in periparturient cows compared with placebo-injected control cows. Data are the reciprocal log2 titer concentration of serum conglutinin ± SEM as determined by a bacterial conglutination assay (n = five cows/group). Data from five Holstein steers bled at the same time averaged seven for all dates (data not shown).

RESULTS

Figure 4. Effect of intramuscular parathyroid hormone (PTH) administration in periparturient cows compared with placebo-injected control cows on specific a) anti-Staphylococcus aureus antibody (data are optical density readings at 405 nm ± SEM determined by enzyme-linked immunosorbant assay. n = five cows/group), and b) anti-Cryptosporidium parvum antibody (data are mean reciprocal titer log2 ± SEM determined by ELISA. n = four cows/group).

DISCUSSION

Results indicated that prevention of periparturient hypocalcemia does not affect hematological changes that occur in periparturient dairy cows (neutropenia, lower serum IgG1 and conglutinin concentrations). Hematologic changes were observed simultaneously in PTH-treated cows and untreated controls, despite a 7-d difference in peak plasma concentrations of 1,25-(OH)2D. Thus, hematologic changes detected at parturition probably are not due to hypocalcemia or hormone fluxes associated with calcium homeostasis. Cortisol concentration in plasma is increased near parturition (7), and this may alter the blood leukogram (22). However, changes in serum immunoglobulin and conglutinin (consistent with immunosuppression existing in vivo) were evident 1 wk before calving, indicating that increased plasma cortisol near calving is not a primary cause of changes in periparturient leukogram, and lower serum IgG1 and conglutinin concentrations. Increased concentrations of cortisol and 1,25-(OH)2D in plasma may contribute to the magnitude and duration of postpartum changes.

The observed decline in total serum IgG1 concentration reported here and by others (1, 12) has several possible explanations. Serum IgG1 concentration will decline due to compartmentalization of IgG1 into cololctogenes, but an impairment of B-lymphocyte function or plasma cell production of IgG1 may also contribute to a decline in serum IgG1 concentration (28). The latter could be due to a direct cellular defect in lymphocytes of the B-cell lineage or to lack of T-
lymphocyte help. In view of the evidence of impaired lymphocyte function at calving (12, 13, 14, 16, 17, 28), compartmentalization of IgG₁ into colostrum does not fully explain the marked decline in serum IgG₁ at calving time. Concentrations of IgM and IgG₂ did not decrease at parturition (which may argue against impaired B lymphocyte function). However, impairment of immunoglobulin secretion by B lymphocytes may be difficult to detect on the basis of serum immunoglobulin concentration, since the half-life of circulating Ig would tend to mask any short-term impairments. Production of IgG₁ by B lymphocytes may be impaired in periparturient dairy cows, but this requires direct immunoglobulin production assays to confirm. Tests for B-lymphocyte secretion of immunoglobulin in cattle were recently developed to specifically address this question (28). Presently, a convincing and complete explanation does not exist for the drop in serum IgG₁ concentration in periparturient cattle.

Serum conglutinin declined at parturition, which is consistent with a previous study where conglutinin in bovine serum decreased near calving (11). The role of conglutinin in disease resistance is not completely known, but the method used to measure conglutinin in this study predicts an increased susceptibility to infection or disease caused by *E. coli* in periparturient dairy cows.

As a further indication of increased susceptibility to disease in periparturient cows, two cows developed clinical mastitis in conjunction with increased bacterial shedding in milk and impaired immunity reported here and previously (17). Shedding of *S. aureus* from the glands of two chronically infected cows seemed to be cyclical during the time period studied. In both animals, *S. aureus* shedding continued to decline after the first episode of clinical mastitis until around 17 or 22 d when *S. aureus* shedding again increased. This time period was associated with onset of estrus and may have provided additional stress to allow increased *S. aureus* shedding. Several studies (2, 10, 24) suggest that a delicate balance exists between subclinical infections and native defense mechanisms. Depletion of peripheral blood PMN numbers (24), delays in entry of peripheral blood PMN into the udder (10), and aflatoxin-induced immunosuppression (2) have all resulted in conversion of subclinical IMI into an acute or peracute mastitis. Clinical mastitis has been suggested to begin with an increase in bacterial numbers in the lacteal secretion (10). The current findings of increased bacterial numbers in lacteal secretions of cows that are leukopenic and have reduced humoral and cellular function (17), along with the appearance of clinical mastitis, support this suggestion.

Impaired immune cell functions were reported in primiparous and multiparous dairy cows at parturition (12, 13, 14, 15, 16, 17, 19, 28). The lactational demands on dairy cows, combined with ruminant metabolism, may be a metabolically demanding phenomenon unique to dairy cows. This results in a negative energy and protein balance in early lactation that may limit the immune system of cows. The appearance of immunosuppression at least 1 wk prepartum suggests that physiologic changes in late gestation adversely influence immunity in dairy cows. Other factors potentially influencing immunity are the changes in estrogen types, concentrations, and ratios to progesterone, which also occur 1 to 2 wk prepartum, coincident with our observed changes in immunity (3, 4, 9).

The most evident effect of periparturient stressors on dairy cows may be a broad-based immunosuppression (affecting both circulating neutrophil numbers and functional activity, lymphocyte function, and humoral components of the native defense mechanisms), thus contributing to the high incidence of clinical mastitis during early lactation. Because hypocalcemic cows are not detectably immunosuppressed more than normocalcemic cows at parturition, we think that the increased risk of clinical mastitis in parturient paretic cows may be the combined result of impaired immune cell functions (which negatively impact cellular and humoral effector mechanisms of immunity) and increased exposure of the teat end to environmental bacteria due to prolonged recumbency of paretic cows.

**ACKNOWLEDGMENTS**

The authors thank Arlen Anderson, Travis Stills, Kim Driftmier, and Bruce Pesch for excellent technical assistance; Chong Hong for statistical analyses; Gene Hedberg for illustrations; and Norm Tjelmeland, Don Robinson, and Jack Moore for animal care.
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