Immunosuppression in periparturient Holstein heifers and cows in the pathogenesis of mastitis

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Immunosuppression in periparturient Holstein heifers and cows in the pathogenesis of mastitis

Kehrli, Marcus Eugene, Jr., Ph.D.

Iowa State University, 1989
Immunosuppression in periparturient Holstein heifers and cows in the
pathogenesis of mastitis

by

Marcus Eugene Kehrli, Jr.

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GENERAL INTRODUCTION

Infectious mastitis begins with the establishment of an intramammary infection (IMI). Bovine mastitis caused by opportunistic environmental pathogens (mostly coliform bacteria and streptococci other than Streptococcus agalactiae) has a high incidence in early lactation. The rates of new IMI caused by environmental pathogens are highest during the first and last 2 weeks of a 60-day, nonlactating period of dairy cows. The rate of IMI during these periods of peak susceptibility is 2 to 12 times higher than any other time. Most coliform and environmental streptococcal infections that are established in the nonlactating period and are present at parturition result in clinical mastitis soon afterward. The proportion of all cases of clinical coliform mastitis that develop during the first 2, 4, and 8 weeks of lactation has been reported to be 25, 45, and 60%, respectively.

Factors contributing to the increased susceptibility to IMI and the progression to clinical disease have not been fully elucidated. Neutrophils can egress rapidly from the blood into the mammary gland in response to an irritant. Phagocytosis and killing of bacteria by neutrophils in conjunction with humoral factors are critical defense mechanisms of the mammary gland. Conditions that may compromise immune mechanisms in the bovine udder include physiologic stress of lactation, a decrease in the number of circulating neutrophils capable of phagocytosis after parturition, dietary aflatoxin, and a delayed udder inflammatory response elicited by bacteria infecting cows in early lactation.
Dystocia, ketosis, and milk fever also have been associated with coliform mastitis after calving.\textsuperscript{15,16}

Investigation of immunosuppression and coliform mastitis in sows revealed depressed neutrophil chemokinesis, ingestion of bacteria, and iodination to be associated with the susceptibility to postpartum mastitis caused by Escherichia coli.\textsuperscript{17} A purpose of the studies reported here was to characterize any alterations in bovine neutrophil function occurring during the periparturient period.

Defects in lymphocyte function also may contribute to the dairy cow's increased susceptibility to mastitis during the periparturient period. Roles for lymphocytes in bovine mammary gland immunity have not been established clearly. Antibody secreted by lymphocytes facilitates phagocytosis. Like macrophages, bovine neutrophils can be activated to have enhanced activity by antigen-induced lymphokines.\textsuperscript{2} Suppressed lymphocyte blastogenesis values after calving, compared with values observed during gestation, have been reported.\textsuperscript{3-5} A purpose of the studies reported here was to characterize any alterations in bovine lymphocyte function occurring during the periparturient period.
The purpose of this review is to present what is known about the pathogenesis of bovine mastitis. This article concentrates on aspects of innate and specific immune defenses involved in protection from mastitis in dairy cattle. The role of immunosuppression in the clinical spectrum of disease is emphasized, proven methods currently available for control are presented and applications of biotechnology to future research on the development of new control methods are outlined.

Introduction

Mastitis is usually an infectious disease of mammals characterized by inflammation of the mammary gland and abnormal lacteal secretions regardless of the cause. Mastitis is a major source of economic loss to the dairy industry, affecting both milk production and milk quality. Bovine mastitis is the most costly disease of American animal agriculture. The National Mastitis Council estimates the cost of mastitis to the dairy industry at about $182 per cow per year, or about $2 billion annually in the United States. A majority of this loss (65 to 70%) is associated with decreased milk yield and thus a lower production efficiency, the remainder of the costs are attributed to treatment. In addition to these direct losses, mastitis causes significant problems in milk quality control, dairy manufacturing practices, quality and yield of cheese, nutritional quality of milk, antibiotic residue problems in milk, meat and the environment, and genetic losses due to premature culling. These additional costs are very significant and are not included in the National
Mastitis Council estimates. In herds with obvious mastitis problems, losses due to mastitis may exceed $300 per cow in the herd.

Much of the current mastitis control and prevention program for the dairy industry depends on drug and chemical usage. Problems related to drug residues and direct health dangers to people handling these chemicals focus our need to reduce the industry's chemical dependency.

Epidemiology and etiology

A total of 137 microbial species, subspecies and serovars have been reported to be isolates from the bovine mammary gland.\(^1\) Historically, the Gram-positive, catalase-positive cocci are the most frequent isolates from the bovine mammary gland. Fourteen of 23 species of the *Staphylococcus* genus have been isolated from the bovine mammary gland. Many of these isolates are not considered to be major mastitis pathogens since coagulase-negative staphylococci have been isolated from 64% of otherwise normal milk samples. Nineteen percent of abnormal milk samples have been found associated with coagulase-negative staphylococci.

Gram-positive, catalase-negative cocci are a long recognized, major group of mastitis pathogens. The most common genera of the Streptococcaceae family isolated from the bovine mammary gland include *Streptococcus agalactiae*, *S dysgalactiae*, *S uberis*, and *S saccharolyticus* (formerly *S bovis*).

Gram-positive, aerobic and facultatively anaerobic bacilli are another major group of organisms isolated from the mammary gland. This group includes minor pathogens (or commensal organisms) such as *Corynebacterium bovis*, and the major pathogens *Actinomyces pyogenes*
(formerly Corynebacterium pyogenes) and *Listeria monocytogenes* which can cause severe mastitis and acute systemic illness.

Gram-negative, facultatively anaerobic bacilli represent a major mastitis pathogen group which are also considered to be opportunists from the environment. Of the Enterobacteriaceae group, the genera *Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Serratia*, and *Proteus* are the most frequent isolates from bovine intramammary infections and present a unique problem as traditional mastitis control methods are generally ineffective. *E. coli* isolates from clinical bovine mastitis have no known virulence mechanisms.2-10

Many other organisms (anaerobes, mycobacteria, spirochetes, mycoplasmas, yeasts and fungi, as well as, the Gram-negative, aerobic and microaerophilic bacilli) have been isolated from mastitic bovine mammary glands. These organisms can cause severe mastitis and economic loss in individual herds or regions. With the possible exception of mycoplasma mastitis in some herds, the prevalence of bovine mastitis caused by these other groups of organisms is low.

There have been major advances in control of the contagious forms of mastitis caused by *S. agalactiae* and *S. aureus* but these methods are costly and entail some public health risk of germicidal and antibiotic residues in milk. The control measures currently employed are largely ineffective against bacteria which cause mastitis through high exposure of teat ends by environmental contamination (coliform bacteria and environmental streptococci).
Environmental mastitis, in general, confronts the dairy industry, veterinarians and mastitis research workers with several complex problems. Detection of environmental organism IMI by microbiological culture of milk is difficult and isolation does not necessarily indicate infection. Prevalence of environmental infections in a dairy herd is usually low even in problem herds experiencing an unacceptable frequency of clinical mastitis. Infections caused by environmental bacteria tend to be of short duration which complicates diagnosis and the assessment of their involvement in mastitis. Surveillance techniques for monitoring mastitis in dairy herds (e.g., milk somatic cell counts) are based on the epidemiology of contagious pathogens and are poorly designed for assessment of environmental mastitis. Eighty to 90% of coliform infections and 50% of streptococcal infections will result in clinical mastitis, and a proportion of the coliform infections will become peracute, thus requiring extensive therapy and may result in a 25% mortality rate.\textsuperscript{11,13,21} The coliform bacteria are highly resistant to most antibiotics approved for use in dairy cows. The clinical symptoms of coliform mastitis are due to an endotoxemia.\textsuperscript{30}

Epidemiological studies on the occurrence of coliform infections and clinical coliform mastitis indicate distinct periods of risk of infection and clinical disease. During the last 2 weeks of a 60-day non-lactating period, dairy cows experience their highest new IMI rate.\textsuperscript{11,12} Most coliform and environmental streptococcal infections established in the dry period which are still present at parturition, result in clinical mastitis soon afterwards.\textsuperscript{13} This period of peak susceptibility to IMI has been
associated with systemic immunosuppression.\textsuperscript{14-19} The proportion of all cases of clinical coliform mastitis which occur, has been reported to be 25\%, 45\% and 60\% during the first 2, 4 and 8 weeks of lactation respectively.\textsuperscript{20-21} Contributing factors in the pathogenesis of coliform IMI and subsequent progression into clinical disease have not been fully elucidated.\textsuperscript{22} Although a causal relationship has not been established, cows with parturient hypocalcemia (PH) have a 5 to 8 times greater chance of having mastitis than if PH is absent and a 9 times greater chance of having coliform mastitis. Parturient hypocalcemia is primarily a disorder of calcium homeostasis, associated with the onset of lactation in dairy cows. Conditions which may compromise immune mechanisms in the bovine udder include: physiologic stress of lactation,\textsuperscript{23} dietary aflatoxin,\textsuperscript{24} and a delayed inflammatory response in the udder elicited by bacteria infecting cows in early lactation.\textsuperscript{25,26} This indicates there are alterations in the native defense mechanisms of the dairy cow around the time of parturition that result in a higher incidence of infection and subsequent clinical disease.

**Innate or non-specific immunity of the bovine mammary gland**

Native defenses of the bovine mammary gland are continually challenged by exposure to bacteria and many factors affect the outcome of this interaction. Establishment of an IMI in dairy cows is dependent upon a delicate balance between native defense mechanisms of the mammary gland and the abilities of bacteria to resist unfavorable growth conditions. The native defense mechanisms of the bovine mammary gland are diverse in nature and range from secretions rich in enzymes and other proteins which
serve to degrade bacterial cell wall components\textsuperscript{27} to cells of the immune system which can act both specifically to ward off infection by a particular microbe (e.g., lymphocytes) and in a non-specific manner to fight bacterial infections (neutrophils and macrophages). Once the teat canal barrier is penetrated by bacteria, the local host defenses in lacteal secretions (lactoperoxidase, complement, lactoferrin and resident immune cells) determine the outcome of bacterial presence in the udder (eradication, subclinical infection or clinical disease).

Neutrophils are one of the most important cell types of native defense mechanisms because they respond quickly (within minutes) and do not require previous exposure to a pathogen to effectively eradicate the microbe. A major function of neutrophils (PMNs) is the phagocytosis and destruction of microorganisms which invade the body. Phagocytosis is probably the most widely distributed defense reaction, occurring in virtually all phyla of the animal world.

The activities of PMNs in combatting microbial infection are complex. Phagocytosis can be broadly interpreted as the combined processes of: 1) PMN movement towards the infection site (or chemotaxis), 2) the recognition and binding of the bacterium to the PMN, 3) engulfment (ingestion) of the bacterium inside the PMN in an inclusion called a phagosome, and 4) the ultimate killing of the bacterium by the various components of the PMN's bactericidal arsenal. Schalm et al., has shown the importance of the neutrophil in protecting the mammary gland against coliform mastitis.\textsuperscript{28-32} Cows made neutropenic with an intravenous administration of an equine anti-bovine leukocyte serum were rendered
incapable of controlling the multiplication of Enterobacter aerogenes. Early and rapid accumulation of sufficient numbers of PMN is paramount in the ability of the host to effect a cure of E. coli infecting the mammary gland.33

Immunosuppression in the pathogenesis of bovine mastitis

Factors affecting the number and functional capacity of neutrophils emigrating into the mammary gland are not clear. In lacteal secretions, PMN ingest milk fat and casein, resulting in degranulation and a loss of pseudopodia, which dramatically reduces the PMN's microbicidal potential.34,35 If milk PMN are derived from already defective blood PMN, or if milk PMN are further impaired by bacterial products, then the balance between bacterial clearance and IMI may favor the development of an IMI or progression of an existing IMI into clinical mastitis. Guidry et al.15 and Newbould14 each have reported alterations in the phagocytic activity of neutrophils at the time of calving. Gunnink reported a deficiency in neutrophil chemotaxis in dairy cows with retained placenta.36

Opportunistic infections, such as the environmental pathogens, are often assumed to be indicative of an underlying condition of immunosuppression in the affected animal. Alterations in non-specific host defenses (such as neutrophil function) or immunosuppression may be quite relevant to the occurrence of new intramammary infections in well managed dairy herds and the subsequent development of clinical mastitis. Immunosuppression associated with gestation and parturition has been reported in many species.37
Recruitment of neutrophils to an infection site is one of the first steps in the inflammatory response. Early and rapid neutrophil influx is important in the eradication of IMI in mice. Defective neutrophil chemotaxis in periparturient cows and diverse abilities of cows to initiate an inflammatory response of neutrophils in the udder during early stages of lactation have been reported. A delay in neutrophil diapedesis into the udder may result in severe disease.

Ingestion of infectious agents is the next step in the phagocytic process. Periparturient changes in yeast and bacterial ingestion by bovine neutrophils have been reported. These studies found either a prepartum increase in the number of yeast cells ingested per neutrophil followed by a rapid decrease at parturition (which was believed to be compensated for by an increase in the percentage of neutrophils phagocytically active), or an increase in the number of phagocytically active neutrophils in blood to a maximum two weeks before calving, which then decreased to a minimum during the first week after parturition. Increased circulating neutrophils have been associated with an increase in blood plasma corticosteroid concentration at parturition. Increased serum cortisol concentration adversely affects bovine neutrophil function and has been implicated as an initiating factor in the pathogenesis of certain bovine infectious diseases.

There have been few reports on changes in later stages of the phagocytic process of neutrophils from periparturient cows. A biphasic response in luminol-enhanced neutrophil chemiluminescence has been
reported. This assay reflects the activity of myeloperoxidase-catalyzed reactions. Enhanced stimulated neutrophil chemiluminescence has been reported in pregnant women, which could not be explained by different amounts of myeloperoxidase in neutrophil granules when compared with controls.40-42

Many neuroendocrine changes develop in cows during the periparturient period. Periparturient hormone fluxes may adversely affect immune cell function. Suppression of human neutrophil chemotaxis by prolactin has been reported in patients with prolactin-secreting tumors.43 There is no effect of estrogen on bovine neutrophil function either during the follicular phase of the estrous cycle in cows or after administration of high doses of estradiol to steers.44,45 However, supraphysiologic concentrations of estradiol have been reported to suppress human neutrophil oxidative metabolism and the neutrophil myeloperoxidase-catalyzed halogenation reaction.46,47 These high concentrations of estrogens may be germane to immunosuppression and the high new IMI rates prior to calving. Before calving, total plasma estrogen concentrations increase in the cow (at least 10 times greater than during estrus).48

Many of the hormonal and metabolic changes that prepare the mammary gland for lactation take place during the 3 weeks preceding parturition. During this critical period, the dairy cow's metabolism shifts from the

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demands of pregnancy to those of lactation, with increased demands for energy and protein. Negative energy and protein balances that exist during early lactation may contribute to impaired neutrophil function and, thus, account for a portion of the periparturient immunosuppression observed.

In lacteal secretions, neutrophils ingest milk fat and casein, resulting in degranulation and a loss of pseudopodia, which dramatically reduces the neutrophils' microbicidal potential. If neutrophils in milk are derived from already defective blood neutrophils, then the balance between bacterial clearance and IMI may favor the bacteria establishing an IMI or the ultimate progression of an IMI into clinical mastitis.

Because mastitis in well managed dairy herds is usually caused by opportunistic bacteria, alterations in non-specific host defenses (e.g., neutrophil function) may be quite relevant to new infection rates and development of clinical disease during the periparturient period. A cause and effect relationship between a faltering immune system and the development of IMI may be difficult to prove, however.

Mitogen-induced polyclonal expansion of lymphocytes has been a useful tool in the study of general lymphocyte function. Mitogen-stimulated lymphocytes produce lymphokines that are varied in function but are known to activate phagocytes and regulate myelopoiesis. Bovine neutrophil function is activated by antigen-induced lymphokine and recombinant bovine g-interferon.
Impaired lymphocyte transformation with mitogens within the first 10 days after calving has been reported. In that study of primiparous and multiparous cows, a decline in PWM response was not observed for primiparous cows. Furthermore, the suppression of responses to PHA and conA was less severe in primiparous cows than multiparous cows. The incidence of mastitis also was correlated with impaired lymphocyte blastogenesis. A severe suppression of bovine lymphocyte responses to PHA within 24 hours of parturition has been shown. Serum and cellular components contributing to an enhanced mitogenic response of lymphocytes during the third trimester of pregnancy have been described. The maximal response of lymphocytes to conA in pregnant heifers at 8.5 months of gestation was reported to be 32% greater than that in an age-matched group of ovariectomized heifers.

A number of investigations have studied the possibility of altered cellular immunity in human pregnancy, but no definitive pattern of lymphocyte function has emerged. Conflicting results on in vitro blastogenesis of lymphocytes from pregnant women may be attributable to various culture conditions and experimental designs. In general, mitogen-induced lymphocyte blastogenesis and cellular immune responses to ongoing bacterial infections are decreased during human pregnancy. Decreased lymphocyte function that persists for 2-3 weeks after delivery has been reported in pregnant women. In another longitudinal study of pregnant women, lymphocyte blastogenesis was found to be decreased during the final 10 weeks of gestation; blastogenesis declined further at delivery. This delivery-associated impairment was attributed to a plasma
factor and a cellular defect similar to findings in cows.\textsuperscript{19,57} Shifts in lymphocyte subpopulations have not been observed in most longitudinal studies to explain the changes in lymphocyte response to mitogen.\textsuperscript{58-62} Basal and stimulated cyclic adenosine 3'-5'-monophosphate concentrations in lymphocytes from women in the final month of gestation are low, compared with those of nonpregnant women.\textsuperscript{63} During normal pregnancy, the progesterone binding capacity of human lymphocytes is increased and the concentration of progesterone in serum during pregnancy are sufficient to reduce the cytotoxic activity of lymphocytes.\textsuperscript{64,65} This raises the possibility that hormone sensitivities of immune cells during gestation may be altered and result in functional changes in immune cells. There is no effect of estrogen on bovine lymphocyte function after administration of high doses of estradiol cypionate to steers.\textsuperscript{45} However, supraphysiologic concentrations of estradiol have been reported to suppress human lymphocyte blastogenesis.\textsuperscript{46,66} High concentrations of estrogens and progesterone are reached during the final days of gestation in cows.\textsuperscript{48} This may be germane to the onset of impaired lymphocyte function in the prepartum cow whose lymphocyte hormone binding capacity may be higher than that in barren cows.

Many neuroendocrine changes develop in cows during the periparturient period. Lymphocyte and neutrophil function could be affected by prepartal increases in estrogens, prolactin, growth hormone, and/or insulin.\textsuperscript{48,67-70} The combination of estrogen, progesterone, growth hormone, and prolactin is known to increase the rate of DNA synthesis in nonlactating bovine mammary tissue grafts.\textsuperscript{71} Whether the bovine lymphocyte responds similarly
is not known. Growth hormone and insulin also are capable of potentiating human lymphocyte responses to mitogen.\textsuperscript{72}

Various degrees of periparturient hypocalcemia are associated with the onset of lactation in dairy cows. This hypocalcemia initiates increases in systemic concentrations of 1,25-(OH)\textsubscript{2} vitamin D.\textsuperscript{81} In humans, 1,25-(OH)\textsubscript{2} vitamin D inhibits mitogen-induced lymphocyte blastogenesis possibly due to inhibition of interleukin-2 production and depressed g-interferon secretion.\textsuperscript{73, 74}

The effect that impairment of circulating lymphocytes may have on local immune defenses in the bovine mammary gland is not known. Milk lymphocytes are hyporesponsive to mitogenic stimulation when compared with autologous blood lymphocytes.\textsuperscript{75} If there is systemic suppression of lymphocyte blastogenesis, it is likely that mammary lymphocytes would be further impaired. The functional role of lymphocytes in milk is not clearly established, but they are capable of antigen-specific clonal expansion.\textsuperscript{76} A deficit in lymphocyte responses to bacterial antigens is likely to reduce the body's resistance to bacterial infections, such as mastitis.

Giesecke suggested that lactating dairy cows are unique in their response to stress, since ruminant metabolism is dependent on glycogenesis/glycogenolysis and lipogenesis/lipolysis for energy-efficient and glucose-sparing feed conversion.\textsuperscript{77} The lactational ability of dairy cows, combined with ruminant metabolism, may be a metabolically-demanding phenomenon unique to dairy cows. The ensuing negative energy and protein balances in early lactation may limit the immune system of cows.
Some of the highest physiologic plasma concentrations of estrogens, progesterone, prolactin, and growth hormone occur in dairy cows during the periparturient period.\textsuperscript{68,70} It is unlikely that periparturient immunosuppression is the result of a change in concentration of a single entity; more likely, it will be found that several entities act in concert with profound effects on the function of many organ systems of the dairy cow. Therefore, it may be very difficult to discern which hormone(s) might contribute to suppression of immune cell function. The most clinically evident effect of periparturient stressors on dairy cows may be immunosuppression, thus explaining the high incidence of clinical mastitis.
Alterations in bovine neutrophil function during the periparturient period.

Marcus E. Kehrli Jr., DVM; Brian J. Nonnecke, MS PhD; James A. Roth, DVM PhD.

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From the U. S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Mineral Metabolism and Mastitis Research Unit (Kehrli & Nonnecke), Ames, Iowa 50010 and the Department of Veterinary Microbiology and Preventive Medicine (Roth), Iowa State University, Ames, Iowa 50011.
ALTERATIONS IN BOVINE NEUTROPHIL FUNCTION DURING THE PERIPARTURIENT PERIOD

Summary

Peripheral blood neutrophils from eight Holstein heifers were evaluated for function during the periparturient period. Random migration, ingestion of bacteria, superoxide anion production, native (non-enhanced) chemiluminescence, iodination and antibody-dependent cell-mediated cytotoxicity by neutrophils were determined. Foremilk samples were evaluated for the presence of bacteria. Significant (P<.05) increases in random migration of neutrophils, iodination, and chemiluminescence were evident 2 weeks before parturition and then decreased dramatically by the first week after parturition. These impairments of neutrophil function after parturition may be manifested as a severe cumulative deficit in the native defenses afforded by the neutrophil.

Introduction

Infectious mastitis begins with the establishment of an intramammary infection (IMI). Bovine mastitis caused by opportunistic environmental pathogens (mostly coliform bacteria and streptococci other than Streptococcus agalactiae) has a high incidence in early lactation. The rates of new IMI caused by environmental pathogens are highest during the first and last 2 weeks of a 60-day, nonlactating period of dairy cows.\(^1,2\) The rate of IMI during these periods of peak susceptibility is 2 to 12 times higher than any other time\(^1\). Most coliform and environmental
streptococcal infections that are established in the nonlactating period and are present at parturition result in clinical mastitis soon afterward.\textsuperscript{1,3} The proportion of all cases of clinical coliform mastitis that develop during the first 2, 4, and 8 weeks of lactation has been reported to be 25, 45 and 60\%, respectively.\textsuperscript{4,5}

Factors contributing to the increased susceptibility to IMI and the progression to clinical disease have not been fully elucidated. Neutrophils can egress rapidly from the blood into the mammary gland in response to an irritant.\textsuperscript{6} Phagocytosis and killing of bacteria by neutrophils in conjunction with humoral factors are critical defense mechanisms of the mammary gland.\textsuperscript{7,8} Conditions that may compromise immune mechanisms in the bovine udder include physiologic stress of lactation,\textsuperscript{9} a decrease in the number of circulating neutrophils capable of phagocytosis after parturition after parturition,\textsuperscript{10,11} dietary aflatoxin,\textsuperscript{12} and a delayed udder inflammatory response elicited by bacteria infecting cows in early lactation.\textsuperscript{13,14} Dystocia, ketosis, and milk fever also have been associated with coliform mastitis after calving.\textsuperscript{15,16}

Investigation of immunosuppression and coliform mastitis in sows revealed depressed neutrophil chemokinesis, ingestion of bacteria, and iodination to be associated with the susceptibility to postpartum mastitis caused by \textit{Escherichia coli}.\textsuperscript{17} The purpose of the study reported here was to determine the alterations in bovine neutrophil function during the periparturient period.
Materials and Methods

Animals and experimental design--Eight healthy Holstein heifers (principals) were evaluated during their periparturient period (ranging from 51 days before to 25 days after parturition). Estrus and breeding were synchronized to minimize the duration of the calving period to be studied. Eight mixed-breed steers were used as controls to adjust for the day-to-day variability typically seen with immune cell function assays. For each immune cell function, the results of the steers were averaged for each day of sampling; then, the results from individual principals for that day were converted to a percentage of the daily average for the controls (percentage of controls). Beginning about 5 weeks before the expected calving time, samples were obtained once a week. The frequency of sampling was increased to a Monday-Wednesday-Friday schedule about one week before expected parturition and continued at that frequency for at least one week after parturition. At that time, the frequency of obtaining samples was reduced to once a week for the next 3 weeks (Table 1).

Bacteriologic examination of milk--Foremilk samples from individual quarters were obtained at the beginning of the experiment and during lactation. Foremilk (0.1 ml) was spread over blood agar plates, which were examined for bacterial growth after 24 and 48 hours' incubation at 39 C.

Hematologic studies--Leukocyte counts in blood were determined by electronic counting of blood collected by jugular venipuncture into tubes containing EDTA. Cytocentrifuge films were prepared and stained with a
combination Wright/Giemsa stain, and > 200 cells were counted to determine the percentage of cell types. Lymphocytes and monocytes were counted together and termed mononuclear cells.\(^\text{18}\)

**Leukocyte preparation**—Neutrophils were separated by hypotonic lysis from packed RBC, as described.\(^\text{19}\) The remaining cells, usually > 95% granulocytes (neutrophils + eosinophils), were suspended to 5.0 \(\times 10^7\) granulocytes/ml in Hanks Balanced Salt Solution without Ca\(^{2+}\) or Mg\(^{2+}\) for functional analysis.

**Neutrophil function assays**—Procedures for evaluating ingestion of \(^{125}\text{I}-\)labeled Staphylococcus aureus,\(^\text{19}\) iodination,\(^\text{19}\) cytochrome C reduction,\(^\text{20}\) and antibody-dependent, cell-mediated cytotoxicity\(^\text{21}\) were performed as described, except that all assays were run at 39 °C and S. aureus strain Newbould 305 (ATCC 29740) was used for neutrophil bacterial ingestion. Tests for neutrophil function were performed in duplicate, except for chemiluminescence, which was performed in single vials (one for resting and one for stimulated chemiluminescence) for each animal. All solutions were prepared with ultrapure, pyrogen-free, double-distilled 18 MΩ\(\text{cm}\) water.

Random migration under agarose by neutrophils was evaluated as reported with modification for data analysis.\(^\text{22}\) A large image of the migration area was traced using a microscope with a drawing attachment, and the area of the image produced was measured with a digitizing pad. Using morphometric analysis, the area of the projected image was mathematically converted to the actual migration area under agarose after the area of the center well was subtracted.
Native (nonluminol dependent) chemiluminescence by stimulated neutrophils was measured using a liquid scintillation counter in the out-of-coincidence mode, with the sample chamber heated to 39 C. The standard reaction mixture for determining stimulated chemiluminescence contained $1.0 \times 10^7$ neutrophils and 2.0 mg of opsonized zymosan (prepared as described in 10 ml of Earles balanced salt solution without phenol red). Nonspecific activation of neutrophils by the media was monitored by omitting opsonized zymosan to determine resting neutrophil chemiluminescence. The reaction was started by adding neutrophils to the vial. Light emission from each vial was measured for 15 seconds at 10-minute intervals over a 90-minute period. Results were converted to the area under the curve.

Statistical analysis--All data analyses were done with the Statistical Analysis System. Results from principals were converted to a percentage of control for each sampling day and then were coded relative to their actual calving dates. Within each week relative to the day of calving (day 0) values from individual principals were averaged and analyzed by fitting the general linear model: $y = \text{mean} + \text{week} + \text{principal} + \text{error}$. In this model, the data were blocked by week (a 7-day period before or after, but not including the day of calving), by principal (representing animal differences), and error represented the residual variation of principals after fitting the model. Statistical significance of differences between week -2 and each of the 3 successive weeks was judged by F tests of the week effect. This analysis was done for both raw data and percentage of control values for the principals.
Percentage of control data were plotted as averages of 2-day periods relative to parturition to evaluate the duration of time when immune function may have changed. Data were analyzed by linear regression to determine the duration of significant changes in normalized principal immune cell function over selected periods. All probabilities were considered significant at the 0.05 level. Three principals were tested on the day they calved. The day-0 data were not included in the plotted weekly averages, but were included in the linear regression analyses.

Results

General observations at parturition--All principals gave birth to a single healthy calf, which was removed from the dam's environment within 8 hours after birth.

Udder health status--After calving, 2 principals (No. 1 and 2) had an E coli IMI. Principal 1 had clinical coliform mastitis 2 days after calving (abnormal milk, fever, mild anorexia, and dehydration). Three quarters were infected and these infections persisted for 2, 14, and 16 days, respectively. Intramammary infection was detected in one quarter of principal 2 at the first milking after calving. Clinical signs of coliform mastitis were not evident. This IMI persisted for 11 days. Both principals recovered without treatment. Intramammary infections caused by coagulase-negative Staphylococcus spp were detected in 1 quarter of each of 3 other principals (No. 3, 4, and 5). One infection was evident at parturition, and the others were detected 2 and 3 days after calving; the infections persisted for 16, 18, and 25 days, respectively. None of the
staphylococcal infections resulted in abnormal milk secretions or signs of illness.

**Periparturient changes in leukogram**--Before calving, total WBC count increased (P < 0.002), which peaked 2 weeks before parturition (Table 2). This increase was caused by the combined absolute increases in the number of circulating neutrophils before calving (P < 0.0001) and circulating mononuclear cells from -6 to -2 weeks before parturition (P < 0.02). At 1 week after calving, the absolute number of circulating neutrophils decreased to about two-thirds the concentration detected one week before parturition.

**Periparturient changes in neutrophil function**--Hypotonic lysis of blood from principals (Table 1) yielded an average of 87% neutrophils (range 77 to 92%). Most of the contaminating cells were eosinophils. Granulocyte purity was 96% for the experiment (range, 95 to 98%). When averages of raw data values from week -2 were compared with those from the 3 following weeks, a significant increase in bacterial ingestion was observed by week -1 (which decreased to values observed in week -2 during week 1, Table 3). A decrease in neutrophil iodination (P < 0.007) was observed during week 1. Resting chemiluminescence values did not indicate nonspecific activation of neutrophils in the assay. Because resting values were essentially constant, stimulated chemiluminescence values were not adjusted. By week 2, stimulated chemiluminescence values were significantly decreased (P < 0.002), whereas iodination values remained low (P < 0.007). During week 1, a decrease in random migration was observed, but was not significant (P < 0.07; Fig. 1).
Comparison of normalized data from week -2 with those from the subsequent weeks revealed general agreement with raw data value comparisons from week 1 (a trend for decreased random migration and significantly decreased iodination). Percentage of control values indicated a trend of depressed cytochrome C reduction that was not significant (P < 0.07). Comparisons of normalized data from week -2 with those from week 2 also agreed with raw data for decreased stimulated chemiluminescence; however, normalized iodination values returned to prepartum values rather than decreasing. Ingestion of bacteria by neutrophils was not significantly changed when analyzed as a percentage of controls.

Linear regressions of normalized neutrophil function values performed on the basis of changes observed in data plots of averages of 2-day periods relative to calving (Table 4). Random migration by neutrophils increased up to 2 weeks before parturition (P < 0.004) and then decreased rapidly until the day of calving (P < 0.006). By the fourth week after calving, neutrophil random migration function had increased and, thus, appeared to recover slightly, but not significantly (P < 0.08). Ingestion of*S. aureus* by neutrophils of principals increased from 7% greater than controls 3 to 4 weeks before parturition to 24% greater than controls for the 4 days before calving. At parturition, ingestion decreased to 11% greater than controls and remained on that level for 2 weeks after parturition. Stimulated chemiluminescence by neutrophils of cows increased from 37% less than controls beginning at day -28 to 60% greater than controls by day -9, but then decreased to 9% less than controls on
the day of calving and remained about the same as controls for 12 days after parturition. One-way analysis of variance comparison of stimulated chemiluminescence for the period between days -18 and -2 with that for the period between days 0 and 10 indicated a significant decrease in the oxidative metabolic capacity of neutrophils of principals during the first 10 days after parturition (P < 0.002). The ability of principals' neutrophils to catalyze the iodination reaction increased from 17% less than controls beginning around day -28 to 24% greater than controls, around day -14 (P < 0.04). From day -14 to day 8, there was a linear decrease (P < 0.002) in the iodination activity of neutrophils. For the first 8 days after calving, neutrophil responses were 15% less than controls in the iodination assay and then recovered. Cytochrome C reduction and antibody-dependent, cell-mediated cytotoxicity by neutrophils did not change significantly during the periparturient period.

Concomitant with the observed increase in certain neutrophil functions about 2 weeks before parturition, there was an increase in eosinophil contamination of the neutrophil preparations (Table 1). The Pearson correlation coefficient between eosinophil contamination of the neutrophil preparations and iodination (r = 0.21) was found to be significant (P<0.02). The correlation coefficient suggests that not more than 4.3% of the variance in the iodination results can be attributed to eosinophil contamination. Because the eosinophil contamination was stable except for week -2, data from that particular week contributed greatly to the correlation.
Immune cell function based on udder health status--As a group, principals with IMI had the most dramatic suppression of the following neutrophil functions: antibody-dependent, cell-mediated cytotoxicity, ingestion, and iodination (Fig. 2). The 2 principals with coliform IMI had more severely suppressed neutrophil antibody-dependent, cell-mediated cytotoxicity, ingestion, and iodination than did the staphylococcal infected group of principals; statistical tests of these differences were not calculated because of the few df.

Correlations between immune cell functions--The principals' neutrophil stimulated chemiluminescence results were significantly correlated (r = 0.43, P<0.0001) with the iodination results. Superoxide anion production measured by cytochrome C reduction also was correlated (r = 0.23, P < 0.02) with the iodination results. All 3 of these assays depend on the oxidative burst of neutrophil metabolism.

Effects of normalizing principals' immune cell function data--The grand mean for each immune cell function assay over the study's duration (Table 5) indicated the controls' immune cells were virtually identical to the overall average of the principals; therefore, the use of the steers' cells to normalize the data for day-to-day variation did not impart any effect of scale on the normalized data from principals. Plots of the controls' raw data values (Fig 3) for the ingestion and iodination values of neutrophil function illustrate a negative slope for the daily values obtained over the course of the experiment.

A components of variance analysis of the raw data and normalized data values of the principals was performed to evaluate the benefit of
normalizing data to block out daily variations in assay results. Raw data variation from one day to the next averaged approximately 4 times greater than variation between principals in 5 neutrophil function assays (Table 5). Conversion of the principals' data into percentage of controls reduced the day effect to the same fraction (0.1) of variance attributed to the animal differences for these neutrophil function assays. On average, normalization reduced the fraction of variance attributed to the day effect for all functions and, therefore, improved the validity of these assays for use in long-term, longitudinal studies. During the 67-day experiment, there was more variation in the principals' cell function than in the controls. Dramatic changes in immune cell function that developed around calving accounted for much of this difference in variation.

Discussion

Primiparous Holstein cows were determined to have impaired neutrophil function after parturition, although peak values of certain neutrophil functions occurred 2 to 3 weeks before parturition. There also was an association of IMI with suppressed neutrophil function in periparturient principals. The association of IMI with impaired neutrophil function is largely based on our findings of immunosuppression and their correlation to a time when mastitis has a high incidence. The zenith and nadir of neutrophil function values observed around parturition are relative to the average values observed during the study. It is possible that the decreased values may reflect a return to normal values. However, based on clinical disease problems after parturition, such as coliform mastitis, we
believe the decrease of neutrophil function after parturition represents an immunosuppressed condition in primiparous dairy cows. Neutrophils of 2 principals with coliform mastitis clearly functioned differently than those of the other principals. The coliform infections were detected after impairment of neutrophil function had been observed. However, we cannot rule out the possibility that coliform mastitis contributed in part to the duration of immunosuppression observed in these 2 principals. Immunosuppression associated with gestation and parturition has been reported in many species.24

Recruitment of neutrophils to an infection site is one of the first steps in the inflammatory response. Early and rapid neutrophil influx is important in the eradication of IMI in mice.25 Defective neutrophil chemotaxis in periparturient cows and diverse abilities of cows to initiate an inflammatory response of neutrophils in the udder during early stages of lactation have been reported.13,14,26 We observed in vitro alterations in neutrophil chemokinesis after calving; the functional significance of which was unclear. Increases in chemokinesis are associated with a loss of neutrophil 'stickiness' and reduced adherence to capillary endothelia.27-29 Adherence is an important initial step when neutrophils egress from the blood stream. Conversely, a dramatic decrease in normal chemokinesis can be interpreted as an impediment to migration through tissues.27,28 A delay in neutrophil diapedesis into the udder may result in severe disease.14

Ingestion of infectious agents is the next step in the phagocytic process. Periparturient changes in yeast and bacterial ingestion by
bovine neutrophils have been reported.\textsuperscript{10,11} These studies found either a prepartum increase in the number of yeast cells ingested per neutrophil followed by a rapid decrease at parturition (which was believed to be compensated for by an increase in the percentage of neutrophils phagocytically active), or an increase in the number of phagocytically active neutrophils in blood to a maximum two weeks before calving, which then decreased to a minimum during the first week after parturition. Results of our ingestion assay indicated that the ingestion capacity of the neutrophil population isolated from blood is high during the periparturient period, but this capacity may decrease slightly after calving. Our data regarding prepartum peaks in total leukocyte numbers, the percentage of neutrophils in blood on the day of calving, and the rapid decrease of neutrophils after calving were in agreement with that reported by Newbould.\textsuperscript{10} Increased circulating neutrophils have been associated with an increase in blood plasma corticosteroid concentration at parturition.\textsuperscript{11} Increased serum cortisol concentration adversely affects bovine neutrophil function and has been implicated as an initiating factor in the pathogenesis of certain bovine infectious diseases.\textsuperscript{9,29,30}

There have been few reports on changes in later stages of the phagocytic process of neutrophils from periparturient cows. A biphasic response in luminol-enhanced neutrophil chemiluminescence, which closely
paralleled our iodination results, has been reported. Both of these assays reflect the activity of myeloperoxidase-catalyzed reactions. The native stimulated chemiluminescence assay is a general measure of neutrophil oxidative metabolism, which detects dismutation of superoxide anion into H₂O₂, myeloperoxidase-catalyzed reactions, and oxidation of membrane phospholipids and arachidonic acid metabolites. That neutrophil functions associated with the oxidative burst of metabolism accompanying phagocytosis are impaired after calving is clearly supported by the correlated depression of stimulated native chemiluminescence and iodination reactions from maximal activity about 2 weeks before parturition to minimal activity the first week after calving. Enhanced stimulated neutrophil chemiluminescence has been reported in pregnant women, which could not be explained by different amounts of myeloperoxidase in neutrophil granules when compared with controls.

Our study found that neutrophil chemokinesis was significantly impaired the first week after calving when compared with activity during week -2. Production of superoxide anion, antibody-dependent cell-mediated cytotoxicity, and bacterial ingestion also decreased during week 1 after parturition, although not significantly.

Many neuroendocrine changes develop in cows during the periparturient period. In our study, the earliest changes in neutrophil function developed 2 to 3 weeks before parturition. Enhanced activities of

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neutrophils before parturition could be attributed to the effects of increases in estrogens, prolactin, growth hormone, and/or insulin before parturition.\textsuperscript{37-41}

Periparturient hormone fluxes also may adversely affect immune cell function. Suppression of human neutrophil chemotaxis by prolactin has been reported in patients with prolactin-secreting tumors.\textsuperscript{42} There is no effect of estrogen on bovine neutrophil function either during the follicular phase of the estrous cycle in cows or after administration of high doses of estradiol to steers.\textsuperscript{43,44} However, supraphysiologic concentrations of estradiol have been reported to suppress human neutrophil oxidative metabolism and the neutrophil myeloperoxidase-catalyzed halogenation reaction.\textsuperscript{45,46} These high concentrations of estrogens may be germane to the onset of impaired neutrophil function in the cow before parturition. Before calving, total plasma estrogen concentrations increases in the cow (at least 10 times greater than during estrus).\textsuperscript{41}

Many of the hormonal and metabolic changes that prepare the mammary gland for lactation take place during the 3 weeks preceding parturition. During this critical period, the dairy cow's body metabolism shifts from the demands of pregnancy to those of lactation, with increased body demands for energy and protein. Negative energy and protein balances that exist during early lactation may contribute to impaired neutrophil function and, thus, account for a portion of the periparturient immunosuppression we and others have observed.
In our study, a group of steers was used to normalize the data from the principals. It was not our objective to use these steers as biologic controls for comparison with the principals. Trends and large day-to-day variations are typical of neutrophil function assays and indicate the need for a control group of cattle to counteract the large day-to-day variation with these assays. A few discrepancies between raw data and normalized data values may arise, as pointed out with the neutrophil iodination values 2 weeks after parturition. This discrepancy points out how use of raw data values from these neutrophil assays can be misleading and erroneous. Significant time trends (sometimes associated with the half-life of radioisotopes used) exist for these cell assays that are, in part, independent of changes in the animals being studied (Fig 3).

In summary, we detected impairment of neutrophil microbicidal mechanisms, and the membrane-associated activities of chemokinesis and ingestion, which may be manifested as a cumulative deficit in the native defense system afforded by neutrophils. Native defenses of the bovine mammary gland are continually challenged by environmental exposure to bacteria, and many factors affect the outcome of this challenge. Once the teat canal barrier is penetrated by bacteria, the local host defenses in lacteal secretions (lactoperoxidase, complement, lactoferrin, and resident immune cells) determine the outcome of bacterial presence in the udder (eradication, subclinical infection, or clinical disease). In lacteal secretions, neutrophils ingest milk fat and casein, resulting in degranulation and a loss of pseudopodia, which dramatically reduces the neutrophils' microbicidal potential. If neutrophils in milk are
derived from blood neutrophils already defective, then the balance between bacterial clearance and IMI may favor the bacteria establishing an IMI or the ultimate progression of an IMI into clinical mastitis.

The results of our study cannot attribute the alterations in the immune status of the periparturient cow to the effect of any specific hormone, combination of hormones, or stress, because these were not evaluated. Because mastitis in well managed dairy herds is usually caused by opportunistic bacteria, alterations in non-specific host defenses (e.g., neutrophil function) may be more relevant to new infection rates and development of clinical disease during the periparturient period. Our results cannot ascribe, however, a cause and effect relationship between a faltering immune system and the development of IMI.

Immunomodulators that might abrogate the development of periparturient immunosuppression currently are not available. Characterization of defective immune cell function during periods of peak IMI rates with opportunistic bacteria should guide efforts to evaluate immunotherapeutic agents for defective bovine neutrophil function.
References


TABLE 1--Frequency of obtaining blood from eight heifers during their periparturient period and purity of neutrophil preparations during this periparturient observational study.

<table>
<thead>
<tr>
<th>Time period</th>
<th>N†</th>
<th>n‡ (%)</th>
<th>(%)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; - Wk -5</td>
<td>4</td>
<td>14</td>
<td>88</td>
<td>9</td>
</tr>
<tr>
<td>Wk -4</td>
<td>6</td>
<td>12</td>
<td>84</td>
<td>11</td>
</tr>
<tr>
<td>Wk -3</td>
<td>5</td>
<td>11</td>
<td>89</td>
<td>9</td>
</tr>
<tr>
<td>Wk -2</td>
<td>7</td>
<td>11</td>
<td>77</td>
<td>21</td>
</tr>
<tr>
<td>Wk -1</td>
<td>8</td>
<td>21</td>
<td>89</td>
<td>8</td>
</tr>
<tr>
<td>Day 0*</td>
<td>3</td>
<td>3</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>Wk 1</td>
<td>8</td>
<td>24</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td>Wk 2</td>
<td>8</td>
<td>18</td>
<td>88</td>
<td>8</td>
</tr>
<tr>
<td>Wk 3</td>
<td>7</td>
<td>8</td>
<td>89</td>
<td>6</td>
</tr>
<tr>
<td>Wk 4</td>
<td>3</td>
<td>3</td>
<td>87</td>
<td>11</td>
</tr>
</tbody>
</table>

*Day 0 represents the day of calving and is not included in the values either the week before or the week after calving. Negative values represent before parturition weeks.

†N = No. of heifers.

‡n = No. of blood samples.
TABLE 2--Regression analyses of selected changes in blood leukograms of eight principals vs time in weeks during the periparturient period.

<table>
<thead>
<tr>
<th>Cells evaluated</th>
<th>Week interval</th>
<th>b*</th>
<th>SE of b</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC count†</td>
<td>-6 to -2</td>
<td>0.016</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>-2 to 3</td>
<td>-0.004</td>
<td>0.002</td>
<td>0.04</td>
</tr>
<tr>
<td>Total mononuclear cell count</td>
<td>-6 to -2</td>
<td>0.006</td>
<td>0.003</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>-2 to 0</td>
<td>-0.01</td>
<td>0.007</td>
<td>0.08</td>
</tr>
<tr>
<td>Total neutrophil count</td>
<td>-6 to 0</td>
<td>0.07</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total eosinophil count</td>
<td>-2 to 0</td>
<td>-0.22</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Mononuclear cells in blood (%)</td>
<td>-6 to 0</td>
<td>-0.004</td>
<td>0.001</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>0 to 4</td>
<td>0.003</td>
<td>0.002</td>
<td>0.19</td>
</tr>
<tr>
<td>Neutrophils in blood (%)</td>
<td>-6 to 0</td>
<td>0.005</td>
<td>0.001</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>0 to 4</td>
<td>-0.004</td>
<td>0.003</td>
<td>0.09</td>
</tr>
<tr>
<td>Eosinophils in blood (%)</td>
<td>-3 to 0</td>
<td>-0.001</td>
<td>0.0006</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0 to 4</td>
<td>0.001</td>
<td>0.0005</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*b - slope of value vs time.
†Linear regressions based on log_{10} cells/mm^3 blood.
TABLE 3--Analysis of variance comparisons of the mean raw data (RD) value and the percentage of controls' (POC) value of a given neutrophil function for all heifers.

<table>
<thead>
<tr>
<th>Neutrophil function</th>
<th>Weekly mean</th>
<th>P-value of week -2 vs week:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2</td>
<td>-1</td>
</tr>
<tr>
<td>Random migration</td>
<td>RD</td>
<td>12.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>139 ± 15</td>
</tr>
<tr>
<td>Antibody-dependent, cell-mediated cytotoxicity</td>
<td>RD</td>
<td>92.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>Cytochrome C reduction</td>
<td>RD</td>
<td>.377 ± .023</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>101 ± 3</td>
</tr>
<tr>
<td></td>
<td>RD</td>
<td>1640 ± 140</td>
</tr>
<tr>
<td>------------------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>Native chemiluminescence§</td>
<td>POC</td>
<td>144 ± 21</td>
</tr>
<tr>
<td>Ingestion‖</td>
<td>RD</td>
<td>59.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>114 ± 3</td>
</tr>
<tr>
<td>Iodination‖</td>
<td>RD</td>
<td>44.3 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>109 ± 7</td>
</tr>
</tbody>
</table>

* Migration area in mm². † Percentage release from [51Cr]-labeled chicken erythrocytes.
† Optical density at 550 nm wavelength. § Mean stimulated area-under-the-curve.
‖ Percentage of 60 Staphylococcus aureus ingested/neutrophil. ¶ nM NaI/10⁷ neutrophils * h.

NS = Not significant at P < .05.
TABLE 4--Regression analyses of neutrophil function values for selected
time intervals, based on averages of 2-day periods* relative to day of
calving (data not shown).

<table>
<thead>
<tr>
<th>Function</th>
<th>time interval</th>
<th>b†</th>
<th>SE of b</th>
<th>PR &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>assay</td>
<td>(Days)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>-47 to -14</td>
<td>0.02</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>random migration</td>
<td>-14 to 0</td>
<td>-0.05</td>
<td>0.02</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>0 to 25</td>
<td>0.02</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>-40 to -14</td>
<td>1.3</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>iodination</td>
<td>-14 to 8</td>
<td>-2.0</td>
<td>0.6</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Only the assays of neutrophil function with significant linear
changes over time are shown.

†Days relative to parturition (i.e., parturition = day 0).

‡Slope of normalized data versus time in days.
TABLE 5--Grand mean comparisons of neutrophil function of principals vs controls over the experiment and a components of variance analysis of the principals' neutrophil function values assuming date and animal effects to be random, independent effects contributing to the overall variation observed in the listed assays.

<table>
<thead>
<tr>
<th>Neutrophil function</th>
<th>Means</th>
<th>Fraction of variance</th>
<th>Random effect</th>
<th>Raw data</th>
<th>Normalized data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steer (SD)</td>
<td>Cows (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody-dependent, cell-mediated</td>
<td>89.3 (0.4)</td>
<td>88.8 (1.7)</td>
<td>Date†</td>
<td>0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>Cytotoxicity*</td>
<td></td>
<td></td>
<td>Animal</td>
<td>0.04</td>
<td>0.06</td>
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<tr>
<td>Chemiluminescence†</td>
<td>1389 (40)</td>
<td>1440 (50)</td>
<td>Date</td>
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<td>0.10</td>
</tr>
<tr>
<td>Ingestion§</td>
<td>54 (0.5)</td>
<td>60 (0.9)</td>
<td>Date†</td>
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<td></td>
<td></td>
<td></td>
<td>Animal</td>
<td>0.09</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Date</td>
<td>Animal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------</td>
<td>--------</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Iodination</td>
<td>37.3 (0.7)</td>
<td>37.4 (1.1)</td>
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<tr>
<td>Chemokinesis</td>
<td>10 (0.4)</td>
<td>10 (0.5)</td>
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<tr>
<td>Cytochrome C*</td>
<td>0.397 (0.004)</td>
<td>0.395 (0.008)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Percentage release from $[^{51}]$Cr]-labeled chicken erythrocytes.

†Average date fractional component of variance for raw data from principals for these assays = 0.27; 4.2 times greater than the animal effect. Normalizing the principals' data with the controls' values each day for these assays reduced the date component to the same fraction of variance (0.1) due to animal differences in these assays. ‡Stimulated area-under-the-curve X 1000. §Percentage of 60 Staphylococcus aureus ingested per neutrophil. ‡‡nM NaI/10$^7$ neutrophil * h. ‡§Migration area in mm$^2$. ‡#Optical density 550 nm wavelength.
Fig 1--Neutrophil function assay results from relative to the day of parturition from 8 Holstein. Bars represent the average difference from the controls for all measurements determined each week on all principals. Weekly standard errors are shown with each bar. ADCC = antibody-dependent, cell-mediated cytotoxicity.
Fig 2---Neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC), bacterial ingestion, and iodination assay results shown by udder health status of 8 Holstein heifers during the periparturient period. Two principals had coliform (Escherichia coli) infections (O-O), three had coagulase-negative Staphylococcus infections (Δ-Δ), and three had no intramammary infections (IMI) detected (•-•). Data points represent the difference from controls by week for the 3 infection status groups during the periparturient period. Weekly standard errors are shown with each point.
NEUTROPHIL ADCC

NEUTROPHIL INGESTION OF S. aureus

DIFFERENCE FROM CONTROLS (%)
Fig 3---Plots of the raw data from 8 controls for neutrophil (PMN) iodination and ingestion of Staphylococcus aureus assays vs the 67 days during which the study was conducted. These were the only two assays with significant negative slopes. Slope (b), SE and P-values for b of each assay is shown. Bars on data points represent the daily SE for the 8 controls.
Alterations in bovine lymphocyte function during the periparturient period.

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Manuscript prepared for publication in American Journal of Veterinary Research

From the U. S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Mineral Metabolism and Mastitis Research Unit (Kehrl Jr. & Nonnecke), Ames, Iowa 50010 and the Department of Veterinary Microbiology and Preventive Medicine (Roth), Iowa State University, Ames, Iowa 50011.
ALTERATIONS IN BOVINE LYMPHOCYTE FUNCTION DURING THE PERIPARTURIENT PERIOD

Summary

Lymphocytes from 8 Holstein heifers were evaluated during the periparturient period for mitogen-induced lymphocyte blastogenic responses. Phytohemagglutinin P- and concanavalin A-induced lymphocyte blastogenesis increased 2 and 3 weeks before parturition, respectively. However, by the first week after parturition, lymphocyte blastogenesis was markedly impaired.

Introduction

The susceptibility of dairy cows to intramammary infections (IMI) and clinical mastitis during the periparturient period, including the associated impairment of neutrophil function, has been discussed.\(^1\) Defects in lymphocyte function also may contribute to the dairy cow's increased susceptibility to mastitis during the periparturient period. Roles for lymphocytes in bovine mammary gland immunity have not been established clearly. Antibody secreted by lymphocytes facilitates phagocytosis. Like macrophages, bovine neutrophils can be activated to have enhanced activity by antigen-induced lymphokines.\(^2\) Suppressed lymphocyte blastogenesis values after calving, compared with values observed during gestation, have been reported.\(^3-5\) The purpose of the study reported here was to determine alterations in bovine lymphocyte function during the periparturient period.
Materials and Methods

Animal experiment design—Eight healthy Holstein heifers (principals) were evaluated during a 67-day periparturient period, as described. Eight mixed-breed steers were used as controls for evaluation of day-to-day variability seen with lymphocyte blastogenesis assays.

Bacteriologic examination of milk—Intramammary infection status of the principals was evaluated with foremilk samples from individual quarters, as described.

Lymphocyte preparation—Lymphocytes were separated by modification of procedures to harvest neutrophils. Anticoagulated blood was centrifuged and the plasma layer was discarded. Five milliliters of the buffy coat layer was removed and diluted in 25 ml of 0.015 M phosphate buffered saline solution (PBSS; pH 7.2). Lymphocytes were enriched by buoyant density centrifugation of diluted buffy coat cells layered over a 3 ml column of 1.084 specific gravity ficoll/sodium diatrizoate in 15 ml conical-bottomed plastic centrifuge tubes at 400 X g for 40 min. The lymphocyte-enriched cells were harvested and washed once in PBSS and then in Hank's balanced salt solution without Ca$^{2+}$ or Mg$^{2+}$ at 400 X g for 10 min. Washed lymphocytes were resuspended to 2 X 10$^6$ cells/ml in RPMI 1640 with 25 mM HEPES buffer and 2 mM L-glutamine.

Lymphocyte blastogenesis—Blastogenic transformation of lymphocytes was measured as described with the following changes. Multiple wells of a flat-bottomed 96-well polystyrene tissue culture plate were seeded with 1.0 X 10$^5$ lymphocytes in a final volume of 0.2 ml RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine. Tissue culture media were
supplemented with fetal bovine serum (heat-inactivated, 12.4% final concentration), 25 units of penicillin G, 6.25 μg of dihydrostreptomycin, and 0.625 μg of amphotericin B/ml of medium. Media for mitogenic stimulation contained either 12.5 μg phytohemagglutinin-P (PHA-P), 7.8 μg concanavalin A (conA) or 2.0 μg pokeweed mitogen (PWM)/ml of medium. After 72 hours at 39 C in a humidified room atmosphere with 5% CO₂, 18.5 KBq of methyl-[³H] thymidine in 0.05 ml of RPMI 1640 medium was added to each well and incubated an additional 18 hours. Well contents were harvested onto glass fiber filters with a cell harvester and the retained radioactivity was determined with a liquid scintillation counter. Lymphocyte blastogenic responses were expressed as the mean counts per minute obtained from triplicate cultures.

**Statistical analysis**—All data analyses were done with the Statistical Analysis System. Results from principals were converted to a percentage of control for each sampling day and then coded relative to their actual calving dates. Individual values within each week relative to the day of calving (day 0) were averaged and analyzed by fitting the general linear model: y = mean + week + principal + error. In this model, the data were blocked by week (a 7-day period before or after but not including the day of calving), by principal (representing animal differences), and error represented the residual animal variation after fitting the aforementioned model. Statistical significance of differences between week -2 and each of the 3 successive weeks was judged by F tests of the week effect. This analysis was done for raw data and percentage of control values for the principals.
Percentage of control data were plotted as 2-day period averages relative to parturition to evaluate the duration of time when lymphocyte function may have changed. Data were analyzed by linear regression to determine the duration of significant changes in normalized lymphocyte function of principals over selected periods. All probabilities were considered significant at the 0.05 level. Three principals were tested on the day they calved. The day-0 data were not included in the weekly averages that are plotted, but were included in the linear regression analyses.

Results

Periparturient changes in lymphocyte function--Comparison of raw data values obtained during week -2 with data obtained during the subsequent 3 weeks indicated a significant decrease in PHAP-induced lymphocyte blastogenesis values during week 1 (Table 1). Lymphocyte blastogenesis induced by conA also was decreased during week 1 (P = 0.04). Normalized data resulted in a conflicting interpretation for PWM-induced lymphocyte blastogenesis values. In one instance (week -1; Table 1), raw data from principals increased when they had actually decreased in comparison with steers' values for the same weeks.

Linear regressions of normalized lymphocyte mitogenic responses vs time were performed on the basis of changes observed in Figure 1 and are in Table 2. There was a significant increase in conA-induced blastogenesis from day -40 to day -18 (P < 0.03). This was followed by a linear decrease in the proliferative response to conA extending from day -18 to day 6, which was statistically significant (P < 0.0001). The
proliferative response of lymphocytes to PHAP peaked at 31% greater than control values about day -10 and then decreased to 27% less than control values by about day 2. The onset and duration of the depressed proliferative response to PHAP was more variable between principals than what was observed for conA; however, the linear decrease in lymphocyte proliferative response to PHAP extending from day -12 to day 2 was significant (P < 0.04). Nonstimulated lymphocytes of the principals incorporated more thymidine than did those of the controls throughout the experiment, possibly indicating a higher basal rate of mitosis. Uptake of thymidine by lymphocytes was increased from about 1.5 times that of controls at 2 weeks before calving to about 5 times that of controls on the day of calving. The reason for this observed increase in the basal rate of mitosis of circulating lymphocytes is not known, but after inspecting the data based on the IMI status (Fig 2), it became obvious that the 2 principals with coliform mastitis contributed greatly to the increase. There was a 1600% increase in the basal mitosis rate of lymphocytes in these 2 principals by week 2.

**Immune cell function based on udder health status**—Cows with IMI had the most dramatic changes in lymphocyte blastogenic responses to conA, PHAP, and PWM stimulation (Fig 2). The 2 principals with coliform IMI had more severely suppressed lymphocyte blastogenesis than the staphylococci-infected group of principals; statistical tests of these differences were not calculated because of the few df.

**Effects of normalizing heifer immune cell function data**—The grand mean over the study for each mitogen used in the blastogenesis assays...
(Table 3) indicates that the controls' lymphocyte blastogenesis values were virtually identical to the overall average of the principals for the experiment, except for the unstimulated cultures. As reported for neutrophil function assays, the use of the controls' cells to normalize the data for day-to-day variation did not impart any effect of scale on the normalized principal results.\textsuperscript{1} Plots of the steer's raw data values (Fig 3) for the mitogenic response of lymphocytes to conA, PHAP and PWM each illustrate a negative slope for the daily values obtained over the course of the experiment. This again indicates the value of a control group of cattle to counteract the trends and day-to-day variation with these assays. A components of variance analysis of the principals' raw data (Table 3) indicates that variation from one day to the next only accounts for 4 and 10% of the total variation resultant from conA- and PHAP-induced blastogenesis measurements, respectively. The day-to-day assay variability for PWM, however, accounted for 58% of the total variation measured during the study. Conversion of the principals' data into percentage of controls reduced the day-to-day variability to only 4% of the total variance, a fraction of the animal effect (26% of the total variation measured) for these assays. Therefore, normalizing the lymphocyte data reduced the day effect and provided a useful technique for evaluating results (e.g., the conflicting results for PWM from week -2 to week -1, Table 3).

Discussion

Our results indicate that primiparous Holstein cows have decreased lymphocyte proliferative responses to the mitogens PHAP and conA after
parturition, compared with peak values of lymphocyte proliferative responses to conA and PHAP 2 to 3 weeks' before parturition. Our results also indicated an association of IMI with suppressed lymphocyte blastogenesis in cows, as reported.9

Immunosuppression associated with gestation and parturition has been reported in many species.10 Our observational study cannot attribute the alterations in blastogenesis of periparturient cows' lymphocytes to the effect of any specific hormone, combination of hormones, or stress, because these were not evaluated.

Mitogen-induced polyclonal expansion of lymphocytes has been a useful tool in the study of general lymphocyte function. Mitogen-stimulated lymphocytes produce lymphokines that are varied in function but are known to activate phagocytes and regulate myelopoiesis.11 Significant correlations between observed alterations in neutrophil function values1 and alterations in lymphocyte blastogenesis values in this study were found. Neutrophil oxidative metabolic capacity measured by the chemiluminescence (r = 0.66, P < 0.001; r = 0.57, P < 0.003) and iodination (r = 0.71, P < 0.0001; r = 0.62, P < 0.001) assays were related to the blastogenic response of lymphocytes to the mitogens conA and PWM, respectively. The strong correlation between lymphocyte blastogenesis and neutrophil oxidative metabolic capacity, could be explained by lymphocyte regulation of neutrophil function via changes in gamma-interferon secretion. Bovine neutrophil function is activated by antigen-induced lymphokine and recombinant bovine γ-interferon.2,12 Another explanation is direct suppression of each cell type by similar or distinct mechanisms.
Whether lymphocytes and neutrophils are directly affected by the stress and neuroendocrine changes associated with the periparturient period or whether neutrophil function is sustained by a lymphokine and is therefore dependent on lymphocyte function during this period cannot be determined from our results.

Impaired lymphocyte transformation with mitogens within the first 10 days after calving has been reported. In that study of primiparous and multiparous cows, a decline in PWM response was not observed for primiparous cows. Furthermore, the suppression of responses to PHA and conA was less severe in primiparous cows than multiparous cows. The incidence of mastitis also was correlated with impaired lymphocyte blastogenesis. In our study, lymphocyte responses to PWM had a large daily variance component compared with that of PHA and conA therefore was a less-sensitive measure of lymphocyte function. Our results indicated a more immediate and marked impairment of lymphocyte responses to conA and PHA that more closely paralleled previous observations on multiparous cows. The higher sampling frequency used in our study during the periparturient period may provide more sensitivity in detecting acute postpartum changes than some previous studies. A severe suppression of bovine lymphocyte responses to PHA within 24 hours of parturition has been shown.

Serum and cellular components contributing to an enhanced mitogenic response of lymphocytes during the third trimester of pregnancy have been described. The maximal response of lymphocytes to conA in pregnant heifers at 8.5 months of gestation was reported to be 32% greater than
that in an age-matched group of ovariectomized heifers; this agrees with our results of a response that is 23% greater than control values at 2 weeks before parturition.

A number of investigations have studied the possibility of altered cellular immunity in human pregnancy, but no definitive pattern of lymphocyte function has emerged. Conflicting results on in vitro blastogenesis of lymphocytes from pregnant women may be attributable to various culture conditions and experimental designs. In general, mitogen-induced lymphocyte blastogenesis and cellular immune responses to ongoing bacterial infections are decreased during human pregnancy. Decreased lymphocyte function that persists for 2-3 weeks after delivery has been reported in pregnant women. In another longitudinal study of pregnant women, lymphocyte blastogenesis was found to be decreased during the final 10 weeks of gestation; blastogenesis declined further at delivery. This delivery-associated impairment was attributed to a plasma factor and a cellular defect similar to findings in cows. Shifts in lymphocyte subpopulations have not been observed in most longitudinal studies to explain the changes in lymphocyte response to mitogen. Basal and stimulated cyclic adenosine 3'-5'-monophosphate concentrations in lymphocytes from women in the final month of gestation are low, compared with those of nonpregnant women. During normal pregnancy, the progesterone binding capacity of human lymphocytes is increased and the concentration of progesterone in serum during pregnancy are sufficient to reduce the cytotoxic activity of lymphocytes. This raises the possibility that hormone sensitivities of immune cells during gestation
may be altered and result in functional changes in immune cells. There is no effect of estrogen on bovine lymphocyte function during the follicular phase of the estrous cycle in cows or after administration of high doses of estradiol cypionate to steers. However, supraphysiologic concentrations of estradiol have been reported to suppress human lymphocyte blastogenesis. High concentrations of estrogens and progesterone are reached during the final days of gestation in cows. This may be germane to the onset of impaired lymphocyte function in the prepartum cow whose lymphocyte hormone binding capacity may be higher than that in barren cows. Various binding capacities also may explain the lack of any estrogen effect on bovine lymphocyte function during estrus or in steers.

Many neuroendocrine changes develop in cows during the periparturient period. In our study, the earliest changes in immune cell function were detected 2 to 3 weeks before parturition. Before parturition, enhanced activities of lymphocytes and neutrophils could be attributed to the effects of prepartal increases in estrogens, prolactin, growth hormone, and/or insulin. The combination of estrogen, progesterone, growth hormone, and prolactin is known to increase the rate of DNA synthesis in nonlactating bovine mammary tissue grafts. Whether the bovine lymphocyte responds similarly is not known. Growth hormone and insulin also are capable of potentiating human lymphocyte responses to mitogen. It is tempting to speculate that the combined effects of these hormones in cows might account for the increased lymphocyte blastogenesis detected 2-3
weeks before parturition and the correlated increase in neutrophil iodination and native chemiluminescence.

Various degrees of periparturient hypocalcemia are associated with the onset of lactation in dairy cows. This hypocalcemia initiates increases in systemic concentrations of 1,25-(OH)₂ vitamin D. In humans, 1,25-(OH)₂ vitamin D inhibits mitogen-induced lymphocyte blastogenesis in part because of inhibition of interleukin-2 production and depressed γ-interferon secretion. Impairment of γ-interferon secretion may contribute to the correlations we found between lymphocyte blastogenesis and neutrophil oxidative metabolism.

In summary, we detected postpartum impairment of lymphocyte function which might predict less than maximal nonspecific activation of neutrophil function by lymphokines. The effect that impairment of circulating lymphocytes may have on local immune defenses in the bovine mammary gland is not known. Milk lymphocytes are hyporesponsive to mitogenic stimulation when compared with autologous blood lymphocytes. If there is systemic suppression of lymphocyte blastogenesis, it is likely that mammary lymphocytes would be further impaired. The functional role of lymphocytes in milk is not clearly established, but they are capable of antigen-specific clonal expansion. A deficit in lymphocyte responses to bacterial antigens is likely to reduce the body’s resistance to bacterial infections, such as mastitis.
References


TABLE 1--Analysis of variance comparisons of the mean raw data (RD) value and the percentage of control steers (POC) values of lymphocyte blastogenesis for all principals.*

<table>
<thead>
<tr>
<th>Lymphocyte stimulus</th>
<th>Mean for week: -2</th>
<th>-1</th>
<th>1</th>
<th>2</th>
<th>-1</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conconavalin A</td>
<td>RD†</td>
<td>243 ± 10</td>
<td>221 ± 8</td>
<td>182 ± 18</td>
<td>252 ± 10</td>
<td>NS</td>
<td>.04</td>
</tr>
<tr>
<td></td>
<td>POC§</td>
<td>107 ± 6</td>
<td>95 ± 2</td>
<td>85 ± 9</td>
<td>119 ± 5</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Phytohemagglutinin P</td>
<td>RD</td>
<td>128 ± 13</td>
<td>95 ± 12</td>
<td>72 ± 9</td>
<td>109 ± 15</td>
<td>NS</td>
<td>.009</td>
</tr>
<tr>
<td></td>
<td>POC</td>
<td>132 ± 17</td>
<td>97 ± 12</td>
<td>72 ± 10</td>
<td>134 ± 18</td>
<td>NS</td>
<td>.01</td>
</tr>
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</table>

*NS: Not Significant
<table>
<thead>
<tr>
<th>Pokeweed mitogen</th>
<th>RD 180 ± 30</th>
<th>215 ± 7</th>
<th>168 ± 14</th>
<th>186 ± 9</th>
<th>NS</th>
<th>NS</th>
<th>NS</th>
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</thead>
<tbody>
<tr>
<td>POC</td>
<td>110 ± 8</td>
<td>105 ± 3</td>
<td>88 ± 6</td>
<td>111 ± 7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* The general linear model of \( y = \text{mean} + \text{week} + \text{principal} + \text{error} \) was used. In this model measures on each principal within a week were averaged for a single value. † Statistical significance was judged by F test of the week effect. ‡ Raw data values are counts (X 1000)/min SEM. § Percentage of control values are based on average week ratios for each principal calculated from arithmetic mean counts/min.

NS = Not significant at \( P < .05 \).
TABLE 2--Regression analyses of lymphocyte blastogenesis values for selected time intervals.*

<table>
<thead>
<tr>
<th>Function</th>
<th>Time interval (days)†</th>
<th>b‡</th>
<th>SE of b</th>
<th>PR &gt; F</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>-18 to 10</td>
<td>13.0</td>
<td>5.8</td>
<td>0.03</td>
</tr>
<tr>
<td>cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA§ stimulated</td>
<td>-40 to -18</td>
<td>1.6</td>
<td>0.7</td>
<td>0.03</td>
</tr>
<tr>
<td>blastogenesis</td>
<td>-18 to 6</td>
<td>-2.4</td>
<td>0.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>PHAP§ stimulated</td>
<td>-12 to 2</td>
<td>-3.5</td>
<td>1.7</td>
<td>0.04</td>
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<tr>
<td>blastogenesis</td>
<td>2 to 14</td>
<td>5.7</td>
<td>2.0</td>
<td>0.008</td>
</tr>
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</table>

*Data were analyzed for selected intervals to determine the duration of significant changes in the principals' normalized lymphocyte blastogenesis.

†Days relative to parturition (i.e., parturition = day 0).

‡b=slope of linear regression versus time in days.

§ConA = concanavalin A, PHAP = phytohemagglutinin P.
TABLE 3--Grand mean comparisons of lymphocyte blastogenesis values of the cows vs the steers for the experiment and a components of variance analysis of the cows' same data assuming day-to-day (Date) and animal-to-animal (Animal) effects to be random, independent effects contributing to the overall variation observed during the study.

<table>
<thead>
<tr>
<th>Lymphocyte stimulus</th>
<th>Means* Controls (SD)</th>
<th>Principals (SD) effect</th>
<th>Variance fraction</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
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<td>Random Raw</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Raw data Normalized data</td>
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<tr>
<td>Unstimulated</td>
<td>0.29 (0.02)</td>
<td>0.73 (1.1)</td>
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<td></td>
<td></td>
<td></td>
<td>Animal 0.22 0.28</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>217 (3)</td>
<td>222 (5)</td>
<td>Date 0.04 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Animal 0.13 0.14</td>
</tr>
<tr>
<td>Phytohemagglutinin P</td>
<td>103 (2)</td>
<td>104 (3)</td>
<td>Date 0.10 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Animal 0.23 0.36</td>
</tr>
<tr>
<td>Pokeweed mitogen</td>
<td>168 (2)</td>
<td>173 (7)</td>
<td>Date 0.58 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Animal 0.10 0.28</td>
</tr>
</tbody>
</table>

*Counts (x1000) per minute of $[^3]H$-thymidine uptake/10$^5$ lymphocytes.

Average Date fractional component of variance for raw data from principals for mitogen-stimulated cultures = 0.24, 1.6 times greater than animal effects. Average Date component of variance for mitogen-stimulated cultures after normalizing principals' data against controls' values each day of the experiment = 0.04, a reduction to 15% of the animal effect.
Fig 1--Lymphocyte blastogenesis assay results from 8 primiparous Holstein cows relative to the day of parturition. Bars represent the arithmetic mean difference by week of the normalized (% of controls) principals' data from controls. Weekly standard errors are shown with each bar. (conA = concanavalin A, PHAP = phytohemagglutinin P, PWM = pokeweed mitogen.)
Fig 2--Lymphocyte blastogenesis assay results from eight primiparous Holstein cows relative to the day of parturition by udder infection status. Two cows had coliform (Escherichia coli) infections (O-O), 3 had coagulase-negative Staphylococcus infections (Δ-Δ), and 3 had no detectable intramammary infections (•••). Data points represent the arithmetic mean difference from controls by week for the 3 infection status groups during the periparturient period. Weekly SE are shown with each point. See Figure 1 for key.
LYMPHOCYTE BLASTOGENESIS

UNSTIMULATED

CON A STIMULATED

NO IMI

STAPH

2000-

200-

-200-

% CHANGE FROM CONTROLS

WEEKS BEFORE AND AFTER CALVING

WEEKS BEFORE AND AFTER CALVING

Weeks 80-2000 Weeks 80-2000

Weeks 80-2000 Weeks 80-2000
Fig 3--Plots of the means of 8 controls' raw data for tritiated thymidine uptake by concanavalin A (conA)-, phytohemagglutinin P (PHAP)-, and pokeweed mitogen (PWM)-stimulated lymphocytes vs the 67 days during which the study was conducted. Slope (b), SE and P value for b of each assay is shown. Bars on data points represent the daily SE for the eight controls.
Periparturient Hypocalcemia in Cows:
Effects on Peripheral Blood Neutrophil and Lymphocyte Function.

Marcus E. Kehrli Jr., DVM; Jesse P. Goff, DVM PhD.

Manuscript prepared for publication in Journal of Dairy Science

From the U. S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Mineral Metabolism and Mastitis Research Unit, Ames, Iowa 50010.
PERIPARTURIENT HYPOCALCEMIA IN COWS: EFFECTS ON PERIPHERAL BLOOD NEUTROPHIL AND LYMPHOCYTE FUNCTION

Summary

Periparturient dairy cows are quite susceptible to intramammary infections and clinical mastitis. Epidemiologic evidence indicates that parturient paresis (milk fever) greatly increases the risk of mastitis, although a causal relationship has not been established. In the present experiment the effects of hypocalcemia at parturition on the immune status of dairy cows were investigated. Ten healthy, multiparous Holstein cows were fed a high calcium diet prepartum to induce hypocalcemia at parturition. Five of these cows received intramuscular parathyroid hormone (crude synthetic N-terminus 1-34) to prevent hypocalcemia at parturition. Effects of hypocalcemia on various neutrophil and lymphocyte functions were determined during the periparturient period, ranging from 6 weeks prepartum to 5 weeks postpartum. All cows exhibited severe loss of immune cell function in the weeks surrounding parturition. Hypocalcemia or the development of parturient paresis did not exacerbate the immune cell dysfunction. This implies that the degree of hypocalcemia observed did not have a large or irreversible influence on neutrophil and lymphocyte function in periparturient cows.

Introduction

The bovine mammary gland during the periparturient period is more susceptible to infection and clinical disease than at other times during lactation or the dry period.\(^1\)\(^-\)\(^3\) Adverse changes in immune cell function
during the periparturient period have been reported, which may contribute to the susceptibility of the bovine mammary gland to infection and clinical mastitis.\textsuperscript{4-10}

Although a causal relationship is not established, epidemiological studies indicate that cows with parturient hypocalcemia (PH) have a 5 to 8 times greater chance of having mastitis than if PH is absent and a 9 times greater chance of having coliform mastitis.\textsuperscript{11} Parturient hypocalcemia is primarily a disorder of calcium homeostasis, associated with the onset of lactation in dairy cows. We hypothesized that changes in plasma calcium concentrations during PH and the associated changes in concentrations of hormones involved with calcium homeostasis might contribute to changes in neutrophil (PMN) and lymphocyte (Lc) function in periparturient cows. If this were true, then prepartum parathyroid hormone (PTH) administration to increase bone resorption and 1,25-dihydroxyvitamin D (1,25-(OH)\textsubscript{2}D) concentrations (which would increase intestinal absorption of calcium), would prevent a decline in plasma calcium concentration at calving\textsuperscript{12} and might alter the adverse periparturient changes in immune cell function. The objective of the present study was to evaluate the ability of PTH treatment and the associated normocalcemia, to restore normal peripheral blood PMN and Lc function during the periparturient period.

Materials and Methods

\textit{Animals and Experimental Design}.--Ten healthy, multiparous Holstein cows were evaluated during the periparturient period ranging from six weeks prepartum to five weeks postpartum. All gestation periods were synchronized to minimize the duration of the calving period to be studied.
As described by Goff et al., all cows received a high calcium diet (about 150 g calcium and 80 g phosphorus/day) prepartum to predispose them to hypocalcemia and milk fever after calving. One group of five cows was given crude synthetic bovine PTH (N-terminal 1-34 fragment, Peninsula Laboratories, Inc., San Carlos, CA) beginning approximately 6.4 d prepartum with a 20 mg dose every 8 h for the first six treatments as a loading dose. Maintenance doses of 10 mg of PTH every 8 h were continued until parturition at which time 20 mg of PTH was administered three times at 8-h intervals. Cows were then gradually withdrawn from PTH therapy by 50% reduction in dose per day down to 2.5 mg/8 h. Withdrawal from PTH was completed after 48 h of treatment at the 2.5 mg dose/8 h (on average by 6.25 d postpartum). Control cows were treated with the carrier alone following this same schedule.

Because normal leukocytes cannot be stored for use as laboratory standards in our function assays, leukocytes isolated daily from five Holstein steers were used as internal laboratory standards to determine the daily mean for each assay. This mean was then used to block out the day-to-day variability typically seen with immune cell function assays by converting the daily individual cow results to a percent of the steers' daily standard mean. Initially, sampling was once a week beginning about 4 weeks before the expected calving time. The frequency of sampling was increased to a Monday, Wednesday, and Friday schedule about two weeks before expected parturition and continued at that frequency for at least two weeks postpartum. The animals were then sampled once a week for the next 2 weeks.
Leukocyte preparation--Neutrophils were separated by hypotonic lysis from packed erythrocytes as previously described. Remaining cells, usually > 95% granulocytes [PMN + eosinophils (PME)], were resuspended to 5.0 \times 10^7 granulocytes/ml in 0.015 M phosphate buffered saline solution for functional analysis. Lymphocytes were isolated as previously described.

Neutrophil function assays--The procedures for evaluating cytochrome C reduction, ingestion of \(^{125}\text{I}\)-labeled Staphylococcus aureus, iodination, native (nonluminol dependent) chemiluminescence, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-independent neutrophil-mediated cytotoxicity (AINC), directed migration and random migration under agarose by PMN were done as described.

Lymphocyte blastogenesis--Lymphocyte blastogenesis was done as previously described with 2.0 \times 10^5 lymphocytes per well. Tissue culture media (0.2 ml of RPMI 1640, Gibco Laboratories, Grand Island, NY, #380-2400) with 25 mM HEPES Buffer and 2 mM L-glutamine was supplemented with the following: heat-inactivated fetal bovine serum (A-1115-L, Hyclone Laboratories, Inc., Logan, UT) to a 10% final concentration and an antibiotic/antimycotic solution (A-9909, Lot 76F-6832, Sigma Chemical Company, St. Louis, MO) resulting in 100 units of penicillin G, 0.1 mg of dihydrostreptomycin and 250 ng of amphotericin B/ml of medium. Media for mitogenic stimulation contained one of the following amounts of mitogen: 25, 12.5 or 6.25 \mu g of phytohemaglutinin-P (PHAP, L-9132, Sigma Chemical Company, St. Louis, MO), 15.6, 7.8 or 3.9 \mu g of concanavalin-A (Con A, C-2010, Sigma Chemical Company, St. Louis, MO), or 20, 2 or 0.2 \mu g of
pokeweed mitogen (PWM, L-9379, Sigma Chemical Company, St. Louis, MO)/ml of medium. Data obtained from all three concentrations of each mitogen were averaged for an overall mitogen mean which was used in statistical analysis.

Data analysis--Data from each cow were converted to a percent of the standard steers' values for each sampling day and then coded relative to their actual calving dates. Standardized values for individual cows were averaged within each week relative to calving. To evaluate the effect of PTH administration on each parameter, week -2 was chosen from Figures 1 and 2, as the pretreatment baseline value for comparison. Changes for each cow from week -2 to each of the following 3 weeks were determined. Average differences between the control cows and the PTH-treated cows were calculated from the individual weekly changes. Any difference different from zero (P<.05) was considered a significant effect of PTH treatment.

To evaluate immunosuppression in all 10 cows across treatments, the individual cow's standardized values within each week relative to the day of calving (Day 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-day 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 week -2 and each of the 3 successive weeks 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 medical intervention; another cow had difficulty standing but recovered without intravenous calcium borogluconate administration. All controls had retained placentae, metritis and were clinically ill. Two cows receiving PTH had twins and both cows developed retained placentae; all other PTH cows cleaned normally. One cow receiving PTH became clinically weak and febrile after calving, was treated symptomatically, and ultimately euthanatized 2 days postpartum. No renal damage nor hepatic damage was evident on postmortem which might have explained her clinical illness. Data from this cow prior to her clinical illness was retained in the data set because the immune cell function results were not disparate from the other cows.

Two cows (one in each group) had experimentally-induced Staphylococcus aureus intramammary infections. Both cows developed clinical mastitis during the first 2 d of lactation. Neither cow was treated for mastitis and both cows were clinically normal by 5 d after calving. Throughout the experiment, data obtained from these cows were not disparate from cows free of intramammary infections and were therefore kept in the data set for analysis.

Periparturient plasma calcium and 1,25-(OH)2D concentrations--Blood calcium levels are shown by Goff et al. In brief, in control cows (prone to milk fever), blood calcium concentrations decreased within 24 h after parturition to below 7 mg/100 ml, whereas blood calcium of cows receiving exogenous PTH was maintained above 9 mg/100 ml. Blood
concentrations of 1,25-(OH)₂D rose significantly in both groups of cows with peak concentrations occurring approximately 7 d earlier in the cows receiving exogenous PTH.

**Periparturient changes in neutrophil function**—Changes in PMN in vitro functions, consistent with the concept of immunosuppression in vivo, were observed in all cows around parturition. In vitro test data of PMN from control cows and PTH-treated cows were virtually identical or parallel throughout the periparturient period despite significant differences in plasma calcium concentration around the time of parturition (Figure 1).

Data from both treatment groups were then analyzed as one group to determine significant changes in PMN function around calving (Table 1). The burst of oxidative metabolism associated with phagocytosis (measured collectively by cytochrome c reduction, native chemiluminescence and iodination assays) in stimulated PMN was significantly impaired during the first two weeks postpartum. Production of superoxide anion by phagocytically active PMN, as measured by cytochrome c reduction was 23 percentage points below the week -2 standardized value for all cows. The iodination reaction measures myeloperoxidase (MPO)-catalyzed halogenation of tyrosine residues present on ingested proteins, in the presence of H₂O₂. This reaction is dependent on adequate production of H₂O₂ (which is formed by spontaneous dismutation of superoxide anion), degranulation of PMN primary granules which contain MPO and the presence of adequate quantities of functional MPO in phagolysosomes. Iodination by PMN was significantly impaired for the first 3 weeks postpartum. Compared with
the standardized value of the cows at week -2, iodination values were 43, 37 and 20 percentage points less the 3 weeks postpartum, respectively. Native chemiluminescence, which is a general measure of oxidative metabolic capacity of PMN, was impaired by 28 and 29 percentage points respectively, the first two weeks postpartum when compared with the standardized values for week -2. The ability of PMN to perform ADCC was also impaired each of the first two weeks after calving by 21 and 39 percentage points below the standardized values during week -2.

Chemokinesis and chemotaxis were not affected in any manner in this group of 10 cows during the periparturient period. Neutrophil bacterial ingestion mediated by Fc receptors was enhanced the week before and after calving by 12 and 10 percentage points, respectively, compared with standardized values during week -2.

It was also evident that some PMN functions were altered prior to calving (Figure 1). Average iodination and ADCC values had decreased by weeks -2 and -1, respectively, by 14 percentage points below the week -3 value. Neutrophil ingestion capacity had also increased 22 percentage points by week -1 from the week -3 value.

Contamination of the PMN preparation by eosinophils is known to affect the results of some PMN assays. Pearson correlation coefficients were calculated between all PMN function parameters and the percent of PME in the PMN preparations. Significant correlation coefficients for PME contamination were found with PMN ADCC ($r = 0.33; p < 0.002$), iodination ($r = 0.80; p < 0.0001$), and PMN directed migration ($r = 0.21; p < 0.05$). Some of the variation in PMN function can therefore be explained by PME.
contamination of the PMN preparations used in the function assays. The percentage of PME contamination ranged from a high of 13% during the fifth week prepartum down to 2% during the second week postpartum, and back up to 10% by the fourth week postpartum. The overall average contamination of PME in the PMN preparations was 8%.

**Periparturient changes in lymphocyte function**—No significant differences in Lc blastogenic responses to mitogens were observed between PTH-treated cows and control cows during or after PTH administration. As shown in Figure 2, data from control cows and PTH-treated cows were virtually parallel throughout the periparturient period in spite of significant differences in plasma calcium concentrations.

Data from both treatment groups were then analyzed as one group to determine significant changes in Lc function around calving time. Significant decreases in mitogen-induced blastogenesis of peripheral blood Lc were detected during the periparturient period in all cows. Compared with standardized week -2 values, Lc mitogenic responses to Con A and PWM were reduced by 22 and 25 percentage points respectively, the week before calving, and responses to PHAP were reduced by 26 and 41 percentage points the weeks before and after calving, respectively (Table 1).

**Discussion**

The data suggest that prevention of hypocalcemia by administration of PTH does not prevent the development of periparturient immunosuppression. In vitro immune cell function changes (consistent with immunosuppression existing in vivo) were evident before calving, indicating that plasma cortisol and 1,25-(OH)_2D concentration increases at calving time appear
not to be the primary causes of periparturient immunosuppression. This was surprising, especially since plasma concentrations of both hormones rose significantly near the time of parturition\textsuperscript{13} and since high concentrations of cortisol in vitro\textsuperscript{16} and in vivo,\textsuperscript{17,18} and of 1,25-(OH)\textsubscript{2}D\textsuperscript{19-20} are known to impair PMN function and Lc blastogenesis. It is quite possible however, that increased cortisol and 1,25-(OH)\textsubscript{2}D levels may have contributed to the magnitude or duration of postpartum immunosuppression.

In other species, 1,25-(OH)\textsubscript{2}D has a variety of effects on immune cell function, including enhanced H\textsubscript{2}O\textsubscript{2} production by phagocytes,\textsuperscript{21} and inhibition of interferon-\gamma,\textsuperscript{20} DNA,\textsuperscript{19} and interleukin-2\textsuperscript{22} synthesis in lymphocytes. Many functional impairments of PMN and Lc in our study, were detected simultaneously, in spite of a seven day difference in peak 1,25-(OH)\textsubscript{2}D plasma concentrations between PTH-treated and control cows. Therefore, we believe the primary cause(s) of immune cell function defects reported here is, most likely, not directly related to hormone fluxes associated with calcium homeostasis.

The data reported here must be considered in the following context: the PMN and Lc in our studies were isolated from normo- and hypocalcemic cows and evaluated under normocalcemlc conditions in vitro. Although the cells were kept in calcium-free media until each assay was conducted, there would be adequate time for extracellular calcium to restore intracellular calcium concentrations (if intracellular pools were decreased in vivo) during the assay. These assay conditions should be
considered when interpreting the severity of immunosuppression which existed in vivo.

If returning the cells to a normocalcemic media influenced the results of these assays, then it is possible that the cows that became hypocalcemic were more immunosuppressed than we were able to demonstrate. Therefore, we cannot conclude with certainty that the in vivo functions of immune cells from hypocalcemic cows were the same as those from normocalcemic cows. Free calcium released into the cytosol of activated cells is derived from intracellular compartments and can occur in the absence of extracellular free calcium. Extracellular calcium probably serves to replete the intracellular pools of calcium, thus maintaining cell activation. However, extracellular calcium concentrations in vitro may not be critical for proliferation of T cells or for PMN random migration. In stimulated hepatocytes, 180 nM extracellular free calcium was sufficient to maintain the release of calcium. Free calcium concentration in dairy cows experiencing PH should not fall below 0.3 mM; therefore, it is unlikely that the degree of hypocalcemia in PH dairy cows is low enough to disrupt cell activation events. It is clear, however, that if cells from hypocalcemic cows are different in vivo, the differences are readily reversed by normal in vitro extracellular calcium concentrations.

The appearance of significant immunosuppression at least one week prepartum suggests that physiologic changes in late gestation adversely influence immune cell function in dairy cows. Estrogen levels increase 1 to 2 weeks prepartum, coincident with our observed development of
immunosuppression.\textsuperscript{27,28} Estrogens have been associated with altered bovine PMN and Lc function.\textsuperscript{29,30} The type of estrogen present may also be important since plasma estrone concentrations are higher than 17-\(\beta\) estradiol concentrations prior to parturition; this contrasts to mainly 17-\(\beta\) estradiol during estrus. Total plasma estrogen in cows at the end of gestation is significantly higher (>100 times) than during estrus and is elevated along with progesterone, which is 4 times greater than progesterone during the luteal phase of the estrous cycle.\textsuperscript{28}

The impaired immune cell functions we observed in multiparous dairy cows at parturition agrees with our earlier findings in primiparous dairy cows.\textsuperscript{7,8} The increase in bacterial ingestion by PMN reported here was also observed in our previous study.\textsuperscript{7} We think this increase in PMN ingestion is associated with defective oxidative killing of ingested bacteria. Because less energy is being consumed by oxidative microbicidal reactions, more energy is available in the PMN to perform ingestion.

We are unaware of reports in other species that describe a parturition-associated immunosuppression that is similar in magnitude and duration to the findings in this and other studies of dairy cows and heifers. Giesecke suggested that lactating dairy cows are unique in their response to stress, since ruminant metabolism is dependent on glycogenesis and glycogenolysis as well as lipogenesis and lipolysis for energy-efficient and glucose-sparing feed conversion.\textsuperscript{31} The lactational ability of dairy cows, combined with ruminant metabolism, may be a metabolically-demanding phenomenon unique to dairy cows. The ensuing negative energy
and protein balances in early lactation may limit the immune system of cows.

In conclusion, some of the highest physiologic plasma concentrations of estrogens, progesterone, prolactin, and growth hormone occur in dairy cows during the periparturient period.\textsuperscript{27,32} It is unlikely that reduced immune cell function is the result of a change in concentration of a single entity; more likely, it will be several entities acting in concert with profound effects on the function of many organ systems of the dairy cow. Therefore, it may be very difficult to discern which hormone(s) might contribute to suppression of immune cell function.

The most evident effect of periparturient stressors on dairy cows may be immunosuppression, thus explaining the high incidence of clinical mastitis. It is evident that PMN are critical in the control of infectious bovine mastitis.\textsuperscript{33} The effect of impaired Lc function on udder health is not well defined, although bovine Lc are known to activate PMN function.\textsuperscript{34} Because hypocalcemic cows are not detectably immunosuppressed more than normocalcemic cows at parturition, we think the increased risk of mastitis in parturient paretic cows is the combined result of impaired immune cell functions, increased exposure of the teat end to environmental bacteria due to prolonged recumbency of paretic cows, and loss of teat sphincter muscle tone.
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blood and bone marrow produced by an equine anti-bovine leukocyte serum. 


TABLE 1—Weekly averages for various neutrophil (PMN) and lymphocyte (Lc) function parameters measured during the periparturient period on 10 multiparous Holstein cows. Data are presented as the mean ± SEM of the standardized data (i.e., percent of the average results of the same 5 Holstein steers measured on each day of the experiment).

<table>
<thead>
<tr>
<th>Immune cell parameter</th>
<th>Weekly mean (± SEM)</th>
<th>P value for Wk-2 vs:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2</td>
<td>-1</td>
</tr>
<tr>
<td>PMN directed migration</td>
<td>98 ± 1</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>PMN random migration</td>
<td>107 ± 6</td>
<td>113 ± 7</td>
</tr>
<tr>
<td>PMN AINC</td>
<td>75 ± 24</td>
<td>68 ± 25</td>
</tr>
<tr>
<td>PMN ADCC</td>
<td>108 ± 7</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>PMN native chemiluminescence</td>
<td>110 ± 4</td>
<td>108 ± 5</td>
</tr>
<tr>
<td></td>
<td>Value1 ± SD</td>
<td>Value2 ± SD</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PMN ingestion</td>
<td>110 ± 4</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>PMN iodination</td>
<td>87 ± 7</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>PMN cytochrome c</td>
<td>98 ± 3</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lc Resting response</td>
<td>69 ± 13</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>Lc ConA response</td>
<td>110 ± 13</td>
<td>88 ± 12</td>
</tr>
<tr>
<td>Lc PHAP response</td>
<td>131 ± 9</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>Lc PWM response</td>
<td>137 ± 9</td>
<td>112 ± 8</td>
</tr>
</tbody>
</table>
Fig 1--Effect of intramuscular parathyroid hormone (PTH) administration on neutrophil functions in periparturient Holstein cows compared with control cows. Data are the difference of the standardized weekly means (n = 5 cows/group) from assay controls derived from 5 Holstein steers. Time of parturition was assigned a zero time value and the weeks represent 168 h time periods before or after parturition.
PTH ADMINISTRATION TO PERIPARTURIENT COWS

S. aureus Ingestion

Control

PTH

Difference from controls (%)  

TIME (weeks relative to calving)

Cytochrome C Reduction

Difference from controls (%)  

TIME (weeks relative to calving)

Native Chemiluminescence

Difference from controls (%)  

TIME (weeks relative to calving)

Antibody-Dependent Cell-Mediated Cytotoxicity

Difference from controls (%)  

TIME (weeks relative to calving)
Fig 2--Effect of intramuscular parathyroid hormone (PTH) administration on lymphocyte blastogenic responses to mitogens in periparturient Holstein cows compared with control cows. Data are the difference of the standardized weekly means (n = 5 cows/group) from assay controls derived from 5 Holstein steers. Time of parturition was assigned a zero time value and the weeks represent 168 h time periods before or after parturition.
PTH ADMINISTRATION TO PERIPARTURIENT COWS

Graphs showing the difference from controls (%) in lymphocyte blastogenesis to CON A, PHAP, and PWM over time (weeks relative to calving).
GENERAL DISCUSSION AND SUMMARY

Bovine mastitis is the most economically important disease in the dairy industry, affecting almost 50% of all cows. The very high incidence of new intramammary infections and clinical mastitis in periparturient dairy cows, as well as the lack of any recognizable virulence factors for many of the pathogens involved, suggested that immunosuppression was involved in the pathogenesis of bovine mastitis. A purpose of our studies reported here was to characterize any alterations in bovine neutrophil function and lymphocyte blastogenesis occurring during the periparturient period.

In the first study, Holstein heifers were determined to have impaired neutrophil function after parturition. Recruitment of neutrophils to an infection site is one of the first steps in the inflammatory response. We observed in vitro alterations in neutrophil chemokinesis after calving; the functional significance of which was unclear. Increases in chemokinesis are often associated with a loss of neutrophil 'stickiness' and reduced adherence to capillary endothelia. Ingestion of infectious agents is the next step in the phagocytic process. Results of our ingestion assay indicated that the ingestion capacity of neutrophils isolated from blood is high during the periparturient period, but this capacity may decrease slightly after calving. After ingesting a microbe, the neutrophil must then destroy the organism. The oxygen-dependent microbicidal mechanisms of the neutrophil are a very potent method of destroying microbes. In our first study we also found that neutrophil functions associated with the oxidative burst of metabolism accompanying
phagocytosis are impaired after calving. This was clearly supported by
the correlated depression of stimulated native chemiluminescence and
iodination reactions from maximal activity about 2 weeks before
parturition to minimal activity the first week after calving. Production
of superoxide anion, and antibody-dependent cell-mediated cytotoxicity
also decreased during the first week after parturition in our first study,
although not significantly.

Our first study results also indicate that Holstein heifers have
decreased lymphocyte proliferative responses to the mitogens PHAP and conA
after parturition, compared with peak values of lymphocyte proliferative
responses to conA and PHAP 2 to 3 weeks' before parturition.

Epidemiologic evidence indicates that parturient paresis (milk fever)
greatly increases the risk of mastitis, although a causal relationship has
not been established. In the second study the effects of hypocalcemia at
parturition on the immune status of dairy cows were investigated. None of
the immune cell function parameters measured in vitro were 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 neutrophil and lymphocyte function in periparturient cows and
that the increased risk of mastitis in the parturient paretic cow is
dependent on some other factor(s). Data in the second study suggest that
prevention of parturient hypocalcemia by administration of PTH does not
prevent the development of periparturient immunosuppression. Neutrophils
and lymphocytes from hypocalcemic control cows and normocalcemic PTH-
treated cows exhibited similar degrees of in vitro dysfunction during the
periparturient period. These assays were conducted in media containing calcium, which may have negated the effects of hypocalcemia on immune cell function in vivo. Therefore, we cannot conclude that immune cell function in vivo is not affected by hypocalcemic conditions, but it is clear that if hypocalcemia causes functional differences in vivo these differences are readily reversed by normocalcemia in vitro.

Because hypocalcemic cows are not detectably immunosuppressed more than normocalcemic cows at parturition, we believe the increased risk of mastitis in parturient paretic cows is the combined result of impaired immune cell functions, increased exposure of the teat end to environmental bacteria due to prolonged recumbency, and loss of teat sphincter muscle tone.

We believe the primary cause(s) of immune cell function defects reported in these two studies is, most likely, not directly related to hormone fluxes associated with calcium homeostasis or to cortisol since immunosuppression was detected prior to the changes in plasma concentration of these hormones. It is quite possible however, that increased cortisol and 1,25-(OH)₂D levels may have contributed to the magnitude and/or duration of postpartum immunosuppression.

The appearance of significant immunosuppression in both studies at least one week prepartum suggests that physiologic changes in late gestation may adversely influence immune cell function in dairy cows. Estrogen levels increase markedly 1 to 2 weeks prepartum, which coincides with our observed development of immunosuppression. It is unlikely that reduced immune cell function is the result of a change in concentration of
a single entity; more likely, it will be several entities acting in concert with profound effects on the function of many organ systems of the dairy cow. Therefore, it may be very difficult to discern which hormone(s) might contribute to suppression of immune cell function.

Significant correlations between observed alterations in neutrophil function values and alterations in lymphocyte blastogenesis values were found. Neutrophil oxidative metabolic capacity measured by the chemiluminescence and iodination assays were related to the blastogenic response of lymphocytes to the mitogens conA and PWM. The strong correlation between lymphocyte blastogenesis and neutrophil oxidative metabolic capacity, could be explained by lymphocyte regulation of neutrophil function via changes in gamma-interferon secretion. Another explanation of these correlated activities is direct suppression of each cell type by similar or distinct mechanisms. Whether lymphocytes and neutrophils are directly affected by the stress and neuroendocrine changes associated with the periparturient period or whether neutrophil function is sustained by a lymphokine and is therefore dependent on lymphocyte function during this period cannot be determined from our results.

Postpartum impairment of lymphocyte function might predict less than maximal nonspecific activation of neutrophil function by lymphokines. The effect that impairment of circulating lymphocytes may have on local immune defenses in the bovine mammary gland is not known. If there is systemic suppression of lymphocyte blastogenesis, it is likely that mammary lymphocytes would be further impaired. A deficit in lymphocyte responses
to bacterial antigens is likely to reduce the body's resistance to bacterial infections, such as mastitis.

Many neuroendocrine changes develop in cows during the periparturient period. In both studies, the earliest changes in immune cell function developed 2 to 3 weeks before parturition. Many of the hormonal and metabolic changes that prepare the mammary gland for lactation take place during the 3 weeks preceding parturition. During this critical period, the dairy cow's body metabolism shifts from the demands of pregnancy to those of lactation, with increased body demands for energy and protein. Negative energy and protein balances that exist during early lactation may contribute to impaired leukocyte function and, thus, account for a portion of the periparturient immunosuppression we and others have observed.

In our studies, a group of steers was used to normalize the data from the principals. It was not our objective to use these steers as biologic controls for comparison with the principals. Trends and large day-to-day variations are typical of neutrophil function assays and indicate the need for a control group of cattle to counteract the large day-to-day variation with these assays. Significant time trends (sometimes associated with the half-life of radioisotopes used) exist for these cell assays that are, in part, independent of changes in the animals being studied. We believe that the use of the steers as internal laboratory standards was a powerful tool which facilitated the interpretation of data obtained from long-term longitudinal studies.

The results of our studies cannot attribute the alterations in the immune status of the periparturient cow to the effect of any specific
hormone, combination of hormones, or stress, because these were not evaluated. Because mastitis in well managed dairy herds is usually caused by opportunistic bacteria, alterations in non-specific host defenses (e.g., neutrophil function) may be more relevant to new infection rates and development of clinical disease during the periparturient period. Our results cannot ascribe, however, a cause and effect relationship between a faltering immune system and the development of IMI.

In summary, we detected impaired lymphocyte blastogenesis and impaired neutrophil microbicidal mechanisms which may be manifested as a significant cumulative deficit in the native defense system. Native defenses of the bovine mammary gland are continually challenged by environmental exposure to bacteria, and many factors affect the outcome of this challenge. Once the teat canal barrier is penetrated by bacteria, the local host defenses in lacteal secretions (lactoperoxidase, complement, lactoferrin, and resident immune cells) determine the outcome of bacterial presence in the udder (eradication, subclinical infection, or clinical disease). In lacteal secretions, neutrophils ingest milk fat and casein, resulting in degranulation and a loss of pseudopodia, which dramatically reduces the neutrophils' microbicidal potential. If immune cells in milk are derived from systemically impaired blood leukocytes, then the balance between bacterial clearance and IMI may favor the bacteria establishing an IMI or the ultimate progression of an IMI into clinical mastitis.

We believe that immunosuppression during the periparturient period, along with changes in the lacteal secretion, predispose the dairy cow to
new IMI and may result in the conversion of these IMI into overt clinical mastitis. Immunomodulation may eventually provide a new tool for management of periparturient dairy cows. Until the factors which alter immune cell function in periparturient cows are understood, the best approach to prevention of periparturient mastitis is to minimize bacterial exposure to the cow by maintaining hygienic conditions in housing areas.
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