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Effect of the presence of the mammary gland on periparturient immunosuppression in dairy cows

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Physiology

Major Professor: Jesse P. Goff

Iowa State University

Ames, Iowa

1999

UMI Number: 9924730

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GENERAL INTRODUCTION

Dissertation Organization

The following dissertation is organized into a general introduction, four journal papers and general conclusions. The general introduction is a review about the literature of periparturient immunosuppression in dairy cows and its associated immunological phenomena. The first paper is a phenotype analysis of leukocyte subset in periparturient dairy cows. The second, third and fourth papers describe the effect of the presence of the mammary gland on the phenotype analysis of peripheral blood mononuclear cells, leukocyte adhesion molecule expression and myeloperoxidase activity in neutrophils, and steroid hormones, respectively. The first paper is published, the third paper is submitted, and the other two papers will be submitted to the Journal of Dairy Science. General conclusions follow the last paper.

Literature Review

Periparturient immunosuppression in dairy cows

It has been empirically determined for a long time that there is a high incidence of infectious and metabolic diseases during the periparturient period in dairy cows and it has been speculated that this high incidence of diseases is associated with impaired immune function. Many researchers have demonstrated the decline in the immune function during the periparturient period compared to non-pregnant and/or non-lactating cows. The impairment of immune function not only increases susceptibility to new infections leading to such diseases as mastitis and metritis (54, 55, 70), but it also can allow subclinical infection by microbes which cause diseases such as salmonellosis and paratuberculosis to become clinical infections.

This high incidence of disease causes a considerable damage to cows and results in a decline in milk production and general health of the herd. Our final goal is to prevent periparturient immunosuppression in dairy cows. In order to reach this goal, we have to know exactly:1) What changes occur in the immune system in dairy cows during periparturient period; and, 2) Why and how periparturient immunosuppression occurs. This literature review focuses on these points and summarizes what we already know about periparturient immunosuppression in dairy cows.

What occurs in immune system in dairy cows during periparturient period?

Immune cell function

Wells et al. (82) showed reduced responses in cultures of lymphocytes from newly calved cows (within 24 hours after parturition) compared to non-pregnant cattle when phytohemagglutinin (PHA) was used as a stimulant. They speculated that this depression in responsiveness of lymphocytes in the immediate postparturient period may be involved in the etiology of disease in cattle during the immediate postparturient period. Since then, many researchers have confirmed the declined lymphocyte function during the periparturient period. Kashiwazaki (30) demonstrated decreased lymphocyte transformation (ability of proliferation) with various mitogens [PHA, concanavalin A(ConA)], and pokeweed mitogen(PWM)) was lowest from parturition to 10 days after parturition, and this impaired function was related to the occurrence of mastitis. He also showed this decreased lymphocyte transformation at parturition was more pronounced in multiparous cows than in primiparous cows which showed a decline in lymphocyte transformation 20 days after parturition rather than around parturition. Kehrli et al. (34) also examined the lymphocyte transformation in primiparous

cows with the same stimuli. Frequent samplings from -5 to 4 weeks after parturition demonstrated a declining pattern of lymphocyte function beginning 2 weeks before parturition and ending 2 weeks after parturition with a nadir at parturition. A similar decline and recovery of lymphocyte function around parturition was reported by Ishikawa et al. (28) in the lymphocyte transformation assay and serum IgG concentration which seemed to be inversely correlated to a serum cortisol level which surged at parturition. Cortisol is a well known immunosuppressant, thus many researchers speculated that periparturient immunosuppression is due to the increased level of cortisol at parturition. A similar pattern of decrease in serum IgG$_1$ levels in addition to a decline in lymphocyte blastogenesis at parturition was also reported by Kehrli et al. (32). This diminished antibody production was confirmed by Nagahata et al. who showed a nadir in the antibody producing activity of lymphocytes at the time of parturition and increase after parturition (52). Diminished lymphocyte function is not limited to these functions. Ishikawa showed a decline in interferon-γ production from -5 to 0 week after parturition and increase after parturition showing nadir during -1 and 0 week (28). Shafer-Weaver showed decreased interleukin-2 and interferon-γ activity, and killing of K562 tumor cell line (this measures the cytotoxic ability of NK cells) in the postpartum cows (within 3 days of calving) compared to mid-lactation (>150 days into lactation) cows (67).

Overall, these findings illustrate that there is an impaired lymphocyte function both in cell-mediated and humoral immunity in periparturient dairy cows. This impaired lymphocyte function is particularly pronounced around parturition when cortisol level is highest, and this seems to be well correlated to the increased incidence of diseases.

Impaired immune function is not only seen in lymphocytes; the neutrophil function also diminishes during the periparturient period. In order to have effective function of neutrophils, it is necessary to have a coordinated process of migration, ingestion, and killing. Periparturient cows showed a diminished ability of these functions except ingestion which showed the highest value at parturition as demonstrated by Kehrli et al. (32, 33). They

speculated this high level of ingestion is due to the decreased killing by oxidation which requires energy. More energy was available for ingestion due to an impaired killing. Whatever the reason is, it is necessary to have coordinated processes of migration, ingestion, and killing. Lack of any of these abilities results in diminished neutrophil function. In their studies, the neutrophil function declined beginning 2 weeks before parturition with recovery beginning 1 week after parturition. A similar pattern was also reported in the phagocytic activity of neutrophils by Saad et. al.(63) and in the chemotaxis of neutrophils found by Nagahata et al. (51). Cai et al. (10) and Kehrli et al. (33) showed this diminished neutrophil function, especially iodination capability, was associated with the increased incidence of periparturient disorders, such as mastitis. The iodination reaction is dependent upon a complex chain of events; including, ingestion, oxidative metabolism, degranulation, and myeloperoxidase activity. Thus, the in vitro iodination reaction is a good screening test to evaluate neutrophil function (60). A decline in both lymphocyte and neutrophil functions was similar and it seemed to be well correlated to the increased incidence of mastitis reported by Smith et al. (70).

Phenotype analysis of immune cells

In order for the immune system to work effectively, the appropriate cell populations should migrate to the appropriate locations. Since the blood stream is the vehicle for immune cells to be carried to the sites needed, it is speculated that periparturient immunosuppression may be associated with the population of circulating leukocytes subsets available to combat new infections.

T-cells play an important role in the immune response by virtue of their ability to recognize antigens with a high degree of specificity, to act as effector cells, and to regulate the nature and intensity of the immune response. T cells are divided into $\alpha\beta$ and $\gamma\delta$ T cells based on the

presence or absence of certain antigenic markers (see Figure 1: classification of immune cells). The proportion of γδ T cells in the circulation of ruminants is far greater than in non-ruminant species, suggesting a unique role for the γδ T cells in ruminant immunology. However, the specific function of the γδ T cells remains unknown. The αβ T-cells are further subdivided into T-Helper and T-Cytotoxic/Suppressor cells. T-Helper cells are instrumental activators of both the humoral and cell-mediated immune systems. They also produce a myriad of cytokines to activate macrophages, lymphocytes, and other cells of the immune system. T-Cytotoxic/Suppressor cells are uniquely equipped to kill tumor cells, parasites, and bacteria. They also produce cytokines to activate and inactivate aspects of the immune response.

The immune cell population analysis revealed the relationship between an inappropriate balance in immune cell subsets and lowered immune function as seen in HIV-infected patients who show decreased percentage and number of CD4 positive cells (T-helper cells, see Figure 2: Immune cells and cell surface markers) and immunodeficiency (41).

The importance of a balance of immune cell proportion rather than the absolute number of immune cells, particularly CD4/CD8 positive cells, is revealed in HIV-infection (4). An association between function andchanges in peripheral blood mononuclear cell (**PBMC**) population is also reported in the dexamethasone induced immune suppression (53). Previous studies of immune cell population in periparturient dairy cows with weekly sampling showed a significant increase in the percentage of circulating CD4 positive cells after calving compared with the percentage of these cells prepartum. Other researchers (56, 77) studied changes in the lymphocyte phenotypes in mammary gland secretions and in PBMC at different stages of lactation. Park et al. (56) showed a significant decline in CD2 (αβ T cells), CD4, and CD8 positive cells (T-Cytotoxic/Suppressor cells) in the mammary gland secretions within 48 h of parturition compared with percentages of these cells at other lactating and non-lactating stages. Taylor et al. (77) found the lowest percentage of CD4 positive cells in mammary gland secretions in early lactation when compared with the percentage of these cells in later stages of

Immune Cell Classification in PBMC
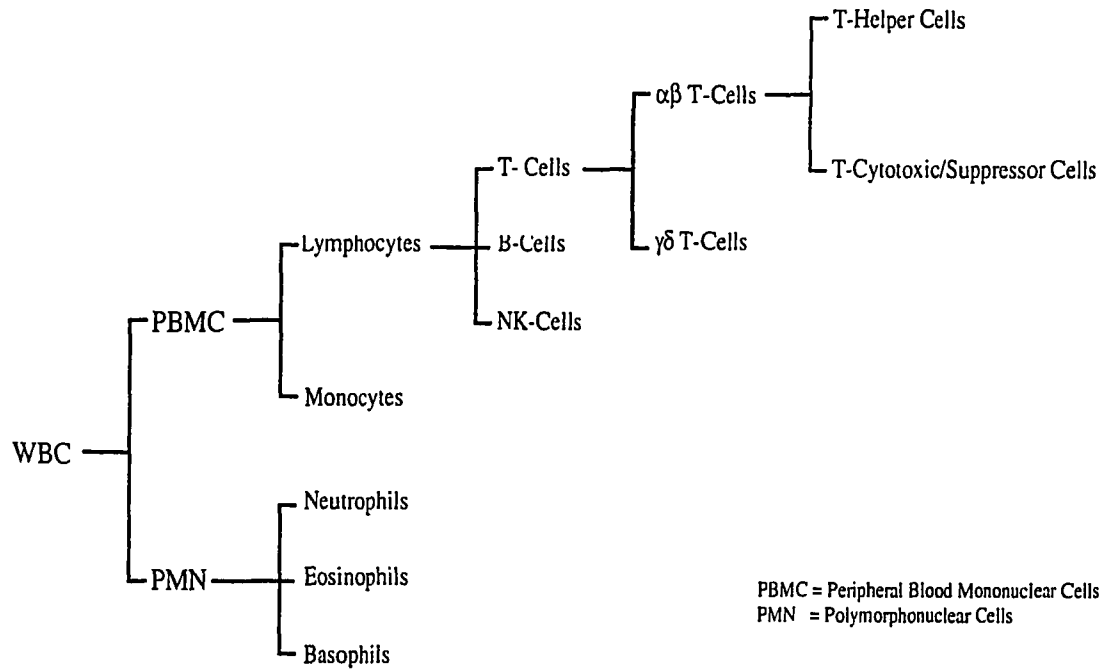
Figure 1: Classification of Immune cells in peripheral blood

Immune Cells and Cell Surface Markers
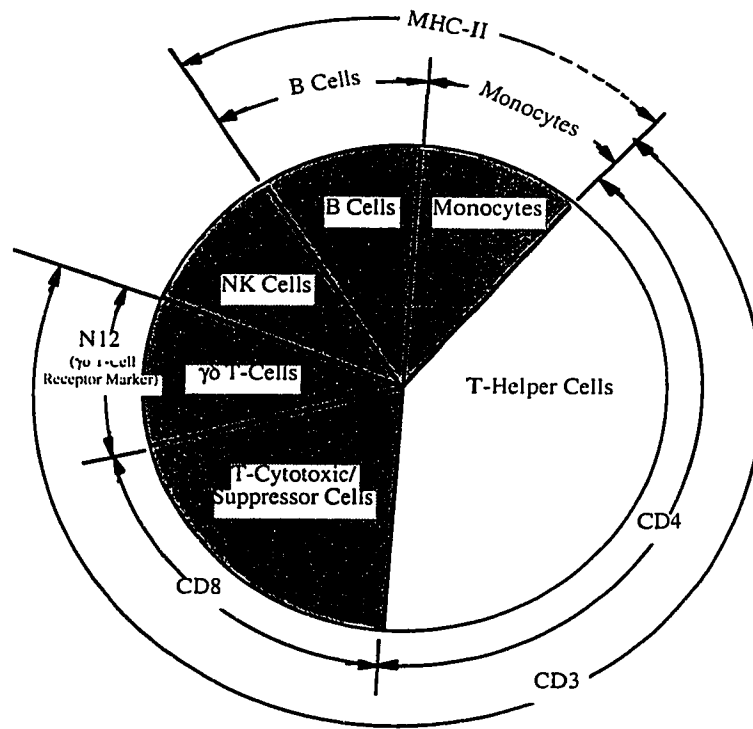
Figure 2: Immune cells and surface markers

lactation. No significant changes in circulating lymphocyte subsets were observed throughout the sampling period in either of the studies. Recently, studies comparing postpartum (within 3 d after calving) and mid-lactation cows (> 150 days into lactation) showed that CD2, CD4, and CD8 positive cells were greatly reduced in PBMC obtained during the postpartum period (67, 68). A similar decline in T cell subsets was seen in the supramammary lymph nodes, and the mammary parenchyma. The decreased T-cell population appeared to be correlated with a diminished function of lymphocytes as determined by various assays (proliferation, interleukin-2 and interferon-γ activity, and PBMC cytotoxicity stimulated by interleukin-2) (67). A monitor of alterations in the proportions of peripheral blood lymphocyte subsets, before, during and after the peripartum period (week -8 to week 16) of dairy cows showed significantly lower T cell subsets at most time points compared to those of non-pregnant, non-lactating cows (29). In this study the lowest value of CD2, CD4, and CD8 positive cells were seen at 3 weeks before parturition with recovery to the initial value by 16 weeks after parturition. The effect of health status (healthy versus infectious, metabolic or reproductive problems) on immune cell population was also examined in this study, however it could not show overall significance on any immune cell subset, which may be due to the low number of cows.

These data suggest that there is a change in the immune cell population, especially a decline in T cell subsets, in periparturient dairy cows, which is thought to be responsible for the diminished immune cell function. However, the time frame studied in these experiments did not precisely define when changes during the periparturient period were occurring.

The information above is based on "normal" cows without experimental infection. What is the response to an experimental infection in periparturient dairy cows? Hill et al. (23) experimentally infected *Escherichia coli* into the mammary gland in newly calved cows which produced a very severe form of mastitis when compared with animals infected in mid-lactation. They showed that a delay in diapedesis of neutrophils into the mammary gland

appeared to be the reason for the subsequent severity of mastitis which showed a peracute state and lack of clinical signs at the beginning of infection. They confirmed that the delayed mobilization of neutrophils and lack of opsonization were responsible for the severity of experimental mastitis in a subsequent study (22). Shuster et al. (69) compared the response to *Escherichia coli* between periparturient (within 10 days after calving), and mid-lactation cows, and showed some similar results. The poor recruitment of leukocyte to the mammary gland was associated with the increased severity of coliform mastitis. In this study, adhesion molecule expression (L-selectin and CD18) on neutrophils was also investigated and the poor up-regulation of CD18 expression seemed to be responsible for the poor phagocytic function and the low recruitment of neutrophils into the mammary gland.

Neutrophils are thought to be an important first line of defense against infection since their response does not require antigen specificity and processing. Thus they can attack invading microbes instantaneously. Immediate recruitment of neutrophils to the site of infection is an important process for the prevention of invasion. In the next section, recruitment of neutrophils into infection sites, specifically the importance of adhesion molecule expression in the recruitment of neutrophils is summarized.

Adhesion molecule expression on neutrophils

Neutrophil migration from blood into an inflamed or infected site is mediated by various adhesion molecules. It requires at least three sequential events. That is, Step 1. Reversible "Rolling" mediated by the L-selectin. Step 2. Activation by chemoattractants in the vascular lumen. and Step 3. Stable binding to the endothelium by $\beta 2$ integrins (8, 11).

Neutrophils are circulating in the blood providing surveillance for invaders. Normally, neutrophils float freely in the blood and do not bind to endothelial cells. In post capillary venules, the blood flow slows down, and neutrophils start rolling on the endothelial cells.

This activity is the "loose tethering" mediated by L-selectin. In the inflammatory process, other selectins, P- and E-selectins are expressed (induced) on endothelial cells in addition to the L-selectin expressed on neutrophils, then they synergistically mediate"rolling"of neutrophils on endothelial cells (37, 73, 78). All these selectins have a lectin domain in their molecules and they bind with carbohydrate on endothelial cells (in the case of L-selectin) or neutrophils (in the case of P- and E-selectins) (78). L-selectin is expressed on neutrophils constitutively and it is thought to be important to promote the initial attachment of neutrophils from the blood stream (capture) prior to the "rolling" which is mediated by all types of selectins (43). The rolling process allows neutrophils to monitor the activating factors in the local environment. Rolling is a reversible process. Without activation by chemoattractants, this loose adherence is releasd and the neutrophils go back to the blood stream to find another site of infection. Neutrophil L-selectin is constitutively functional and is expressed at high levels on circulating, resting neutrophils. It is capable of mediating attachmentof neutrophils to endothelial cells under shear flow conditions without neutrophil activation. The affinity of L-selectin for ligands can be increased after stimuli (21, 73, 74).

The second step is "activation" by chemoattractants, proinflammatory mediators. Chemoattractants are associated with or released from the endothelial cell surface during the inflammatory process and they can activate neutrophils leading to tight adhesion, thus arrest of neutrophils on endothelial cells. Chemoattractants include, C5a, a product of complement activation; leukotriene B4, an arachidonate metabolite; and platelet-activating factor, a product of phosphatidylcholine metabolism. Chemokines, such as interleukin-8, are also chemoattractants (72). These chemoattractants bind with receptors on neutrophils and activate them. This activation causes a transient increase in the affinity of L-selectin to ligands on endothelial cells, then it leads to shedding of L-selectin by protease activity (37). Conversely, this activation by chemoattractants increases the function and surface expression of the integrins expressed on neutrophils (11, 75). Now neutrophils adhere tightly to endothelial

cells with this increased expression of integrins and their ligands, intracellular adhesion molecule (ICAM)-1 and 2.

The third step is "tight adhesion" or "arrest" of neutrophils to endothelial cells mediated by integrins. The integrins expressed on neutrophils are $\beta2$ integrins. The $\beta2$ integrin family consists of distinct α chains, CD11a, CD11b, and CD11c, and these integrins are noncovalently linked with a common β chain (CD18). Neutrophils express all three kinds of $\beta2$ integrins. They are also stored in secondary and tertiary granules in the neutrophils and their expression increases after stimulation by chemoattractants, especially CD11b/CD18 (9, 26, 75). In the resting state, they do not adhere tightly to other cells. Activation changes the conformation of the integrins which enables the binding to their ligands, however, increased expression of $\beta2$ integrins on neutrophils is neither necessary nor sufficient to promote neutrophil aggregation (9, 57, 79). Stimulation by chemoattractants and divalent cations are necessary for $\beta2$ integrins to be functional (3, 9).

After the arrest of neutrophils on the endothelial cells with tight adhesion of $\beta2$ integrins to their ligands, ICAM-1 and 2, neutrophils migrate tbetween the endothelial cells by the homotypic interaction of platelet endothelial cell adhesion molecule 1 (**PECAM-1**). PECAM-1 is present on endothelial cells at sites of intercellular junctions and it is also expressed by neutrophils. PECAM-1 is capable of interacting with itself, which leads to the transmigration of neutrophils (1, 11).

The key feature of neutrophil migration into the site of information (inflammation) is that, the three steps mentioned above occur in sequence, not in parallel. The inhibition of any one of these steps gives essentially complete, rather than partial, inhibition of neutrophil migration (74).

What happens to these adhesion molecules in periparturient dairy cows?

A study with 8 Holstein cows during periparturient cows showed an increase in CD18 expression toward parturition while at the same time showing the highest expression at

parturition both in constitutive and platelet-activating factor induced expression (42). This CD18 expression dropped during the first 24 hours after parturition and returned to prepartum values by day 3 after parturition. Conversely, L-selectin expression decreased markedly by 9 to 24 hours after parturition, and returned to prepartum values by day 3 after parturition. Following the decrease in L-selectin expression, circulating neutrophils increased at parturition and they returned to prepartum value after parturition. This pattern of change in L-selectin expression and neutrophil number in circulation seemed to be well correlated to the increase in cortisol level at parturition. The effect of glucocorticoids on expression of CD18 and L-selectin showed a similar pattern seen in periparturient cow study, i.e., down regulation of both L-selectin and CD18 expression by dexamethasone (6, 7). Therefore, it is speculated that an increased cortisol level at parturition causes delayed migration of neutrophils into ites of inflammation. This is one of the reasons cows are susceptible to infectious diseases at parturition.

Why and how periparturient immunosuppression occurs?

The reason for periparturient immunosuppression is still unknown but there are several possible explanations. Cows during the periparturient period are exposed to tremendous physiological changes associated with parturition and colostrum production. Both of these factors could cause immunosuppression since both processes are associated with increased nutrient requirement and changes in hormone profiles.

Effects of nutrition on immune function in dairy cows

Dairy cows are known to have a negative energy balance during the periparturient period since feed intake does not meet the requirement for the final growth of the fetus and for

colostrum synthesis. The plasma non-esterified fatty acid concentration increases prior to and at parturition (20) which indicate a negative energy balance. Adequate nutrition is required to maintain a healthy immune system. It is reported that calorie restriction impairs immune functions, for example it alters macrophage function due to impairment in signal transduction (58, 59), and modulates lymphocyte subset phenotype due to increased apoptosis (45). Nutritional modulation enhances the susceptibility to infectious diseases such as mycobacterial infections (14, 47). Cows during the periparturient period are not only susceptible to metabolic diseases such as ketosis and milk fever, but also to infectious diseases. The metabolic diseases may increase the susceptibility to infectious diseases. It is reported that milk fever cows have higher susceptibility to coliform mastitis (13), and ketosis is associated with an increased severity of experimental *Escherichia coli* mastitis (38). However, Kehrli et al. showed no significant difference in immune function of either PBMC or neutrophils between milk fever and non-milk fever cows (31). Franklin et al. also could not show the effects of ketones on in vitro lymphocyte proliferation (16). These discrepancies may be due to the timing and frequency of samplings and/or the sensitivity and nature of assays. Calorie restriction as well as other nutrient-insufficiency causes immunosuppression (40). In addition to a negative energy balance, dairy cows in periparturient period suffer from lower levels of vitamins and minerals (18). The relationship between vitamins, minerals and the immune system has been studied (17). Significant reports have been published in this area. One of them is the special issue of Nutrition Review (46) which summarizes the lectures presented at the "Nutrition and Immunity" conference held at Atlanta, Georgia in 1997. Although cows during the periparturient period tend to suffer from negative energy and imbalances of mineral and vitamin level, it is not known if this condition is the result of growth of the fetus toward the termination of gestation or the result of colostrum production.

Effects of steroid hormones on immune function in periparturient dairy cows

Cows, during the periparturient period, are also exposed to tremendous changes in neuroendocrine system associated with parturition and colostrum production. Among these are significantly higher levels of steroid hormones (progesterone, estrogens, and cortisol) during this period. Estrogen (both estrone and estradiol) levels increase markedly at the end of gestation starting from the last 3 week of gestation and reaching a zenith at parturition (65, 71). The progesterone level remains high during pregnancy, then it drops precipitously just prior to calving (71). The cortisol level surges around parturition (d -2 to 2) (71). High levels of these steroid hormones are known to have immunosuppressive effects both in humoral and cell-mediated immunity (19, 48, 61, 62, 64, 76); thus they are thought to be responsible for periparturient immunosuppression in which both humoral and cell mediated immunity are suppressed (27, 32, 52). Cows are immunosuppressed during pregnancy as are other mammals in order to maintain the "foreign tissue, fetus" inside the body (44). This pregnancy-associated immunosuppression is thought to be due to the high levels of estrogen and progesterone. There are reports which describe the relationship between high level of estrogen and progesterone and maternal immunosuppression (36, 39, 64, 80). Both of these hormones are known to have immunosuppressive effects both in neutrophils and PBMC (19, 50, 76, 81). Periparturient immunosuppression is "additional" to this pregnancy-associated immunosuppression. Studies of immune function in periparturient dairy cows showed an almost linear decline in both neutrophil and PBMC function (27, 33, 34, 52). These changes seem to be negatively correlated to the increase in estrogens; therefore it is speculated that estrogens are responsible for the immunological impairment.

Cortisol is the most well known immunosuppressive steroid. There are extensive studies about the relationship between high cortisol levels and diminished immune cell function (2, 5,

27, 49, 61, 66). Although a surge of cortisol is seen only at parturition, one point sampling at parturition leads us to believe a close relationship between immunosuppression and high level of cortisol. In an adhesion molecule study with frequent samplings around parturition (42), a sudden increase in cortisol seemed to be associated with the down regulation of L-selectin, hence the increase in circulating neutrophil numbers.

Mammogenesis and colostrogenesis require the secretion of many kinds of hormones in addition to nutrition (25). A high level of steroid hormones induces colostrogenesis, but these steroid hormones are also required for parturition. It is not known how these steroid hormones contribute to the periparturient immunosuppression since other peptide hormones, such as growth hormone and prolactin are also involved in colostrum production. These hormone levels increase toward the time of parturition (12) and they are known to have immunomodulatory effects (15, 24, 35).

Conclusion

Increased susceptibility of cows to diseases during the periparturient period is thought to be due to the impaired immune function of PBMC and the neutrophils. Although these changes seem to be associated with changes in immune cell subsets, especially the decrease in T cell population, it has not known when and how immune cell population changes during the periparturient period. The reason for this periparturient immunosuppression is still unknown, although negative energy, minerals, and vitamin balance and increased steroid hormone levels, which are associated with parturition and colostrum production, seem to contribute to immunosuppression. Moreover, it is not known how milk production contributes to these phenomena. Our research hypotheses were:

1) Periparturient immunosuppression is closely associated with changes in circulating immune cell population.

2) Milk production plays an important role in periparturient immunosuppression both for PBMC and the neutrophils.

3) Increases in steroid hormones associated with parturition and colostrum production contributes to the periparturient immunosuppression.

In order to prove these hypotheses, we did several experiments and summarized them in the four chapters of this dissertation.

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PHENOTYPE ANALYSIS OF PERIPHERAL BLOOD MONONUCLEAR CELLS IN PERIPARTURIENT DAIRY COWS

A paper published in the Journal of Dairy Science [1]

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ABSTRACT

Impaired immune function during the periparturient period contributes to the increased susceptibility of the cow to infectious disease around the time of calving. Changes in subpopulations of peripheral blood mononuclear cells during the immediate periparturient period

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can contribute to the observed immunosuppression in cows but it is not known exactly when and what changes occur. Using a flow cytometer and monoclonal antibodies directed against antigenic markers on mononuclear cells, the populations of CD3, CD4, CD8, and γδ-T cell receptor positive cells were examined in eight periparturient Jersey cows during 2wk before and after parturition. The percentage of cells that were positive for CD3, CD4, and γδ-T cell receptor markers exhibited a significant decline before calving and reached a nadir at calving. These percentages did not return to precalving levels until 2 wk after calving. These data are compatible with the hypothesis that declining T-cell populations may contribute to the immunosuppression reported for dairy cows at calving.

(**Key words:** immunosuppression; periparturient; lymphocyte; CD4, CD8, and γδ-T cell receptor positive cells)

Abbreviation: PBMC = peripheral blood mononuclear cells

INTRODUCTION

The increased incidence of infectious disease, especially mastitis, in periparturient dairy cows has been associated with impaired neutrophil and lymphocyte function during the immediate peripartum period (2, 10, 11). We suspected that changes in immune function in periparturient dairy cows might be reflected in changes in T-cell subset populations in peripheral blood mononuclear cells (**PBMC**).

T-cells play an important role in the immune response by virtue of their ability to recognize antigens with a high degree of specificity, to act as effector cells, and to regulate the nature and intensity of the immune response. T cells are divided into αβ and γδ T cells based on the

presence or absence of certain antigenic markers. The proportion of $\gamma\delta$ T cells in the circulation of ruminants is far greater than in non-ruminant species, suggesting a unique role for the $\gamma\delta$ T cells in bovine immunology. However, the specific function of the $\gamma\delta$ T cells remains unknown. The $\alpha\beta$ T-cells are further subdivided into T-Helper and T-Cytotoxic/Suppressor cells. T-Helper cells are instrumental activators of both the humoral and cell-mediated immune systems. They also produce a myriad of cytokines to activate macrophages, lymphocytes, and other cells of the immune system. T-Cytotoxic/Suppressor cells are uniquely equipped to kill tumor cells, parasites, and bacteria. They also produce cytokines to inactivate aspects of the immune response. Decreases in both the total number and relative percent of peripheral blood T-Helper cells have been used as an indicator of compromised immune function. For example, human patients with acquired immunodeficiency syndrome (AIDS) commonly have decreased numbers of T cells and an inversion of T-Helper/T-Cytotoxic/Suppressor cell (CD4/CD8) ratio (4, 9).

In a previous study using cows that were sampled weekly around parturition, Harp et. al.(7) found a significant increase in the percentage of circulating CD4 positive cells (T-Helper cells) after calving compared with the percentage of these cells prepartum (6). Other researchers (12, 15) studied changes in lymphocyte phenotype in mammary gland secretions and in PBMC at different stages of lactation. Park et al. (12) showed a significant decline in CD2 ($\alpha\beta$ T-cells), CD4, and CD8 positive cells (T-Cytotoxic/Suppressor cells) in mammary gland secretions within 48 h after parturition compared with the percentages of these cells at other lactating and nonlactating stages. Taylor et al. (15) found the lowest percentage of CD4 positive cells in mammary gland secretions in early lactation compared with the percentage of these cells in later stages of lactation. No significant changes in circulating lymphocyte subsets were observed throughout the sampling period in either of those studies. Recently, studies comparing postpartum (within 3 d after calving) and midlactation cows showed that CD2, CD4, and CD8 positive cells were greatly reduced in PBMC obtained during the

postpartum period (13, 14). The decreased T-cell population appeared to be correlated with diminished function of lymphocytes as assessed by various assays (proliferation, interleukin-2 and interferon-γ activity and cytotoxicity stimulated by interleukin-2) (13).

In this study, total T-cell, T-Helper , T-Cytotoxic/Suppressor, and γδ T-cell populations were examined using monoclonal antibodies against CD3, CD4, CD8 and N12 markers (3) respectively with a more intensive sampling frequency to determine exactly when and what changes occur in these cell subset percentages during the periparturient period.

MATERIALS AND METHODS

Cows

Eight multiparous Jersey cows between 5 and 8 yr of age were used in this study. Blood samples were collected by jugular venipuncture into tubes containing the anticoagulant acid citrate dextrose and ethylenediaminetetraacetic acid on d -13, -10, -8, -6, -5, -4, -3, -2, -1, and 0 (within 3 h of calving) and d 0.5, 1, 2, 3, 4, 5, 6, 8, 10, and 13 after calving.

Cell Preparation
and Antibody Binding

Whole blood collected in tubes containing anticoagulant acid citrate dextrose was centrifuged at 1800 X g for 7 min. The buffy coat was harvested and diluted to approximately the original blood volume with PBS (pH 7.2) solution. Fifty microliters of this cell suspension were then incubated with 50 μl of primary monoclonal antibody (described subsequently) at 4°C for 30 min in a microtiter plate (U bottom; 96 well). The supernatant was decanted after centrifugation at 800 X g for 4 min. The cells were then treated with 150

μl of hypotonic lysing solution (1) for 90 s at room temperature (around 22°C) to lyse erythrocytes. Then, 75 μl of hypertonic restoring solution (1) were added to restore isotonicity. The supernatant was decanted after centrifugation at 800 X g for 4 min. The lysing process was repeated once more. The remaining leukocytes were washed once with 200 μl of PBS and incubated at 4°C for 15 min with 50 μl of the secondary antibody described subsequently. After incubation and a single wash with PBS, the cells were suspended in 200 μl of sheath fluid (Isoton® II; Coulter Diagnostics, Hialeah, FL) for immediate flow cytometric analysis.

Antibodies

The primary antibodies used were all specifically reactive to bovine markers (8), and descriptions of working solutions are presented in Table 1. The secondary antibodies used were FITC-conjugated goat anti-mouse IgG (H+L) F(ab)′$_2$ (Caltag Laboratories, San Francisco, CA) or PE-conjugated goat anti-mouse IgM (H+L) F(ab)′$_2$ (Southern Biotechnology Associates, Inc., Birmingham, AL) which were diluted 1:100 or 1:500 (vol/vol), respectively, in phosphate buffered saline with 1% fetal bovine serum for use as a working solution.

Flow Cytometric Analysis

Data from 5000 events per sample were acquired (Cell Quest software; Becton Dickinson, San Jose, CA) using a FACScan flow cytometer. For all analyses (Cell Quest software), mononuclear cells were gated out from granulocyte populations based on their forward and side scatter characteristics on density plots (1). Mouse IgG$_1$ and mouse IgM (Sigma Chemical Co., St. Louis, MO) were used as isotype controls to define a baseline reference point. Cells with higher fluorescent intensity than this reference point were considered positive. Marker positive cells are expressed as a percentage of PBMC.

Enumeration of Peripheral Blood Mononuclear Cells

Total leukocyte counts were determined with whole blood collected in tubes containing

ethylenediaminetetraacetic acid using an electronic cell counter (CellTrack; Angel Engineering

Corp., Trumbull, CT). Slides for differential cell counting were prepared by

cytocentrifugation (CytoSpin; Shandon, Sewickley, PA), stained with a combination

Wrights/Giemsa stain (StatStain; VOLU-SOL, Henderson, NV), and 100 cells were counted

to determine the number of mononuclear cells.

Statistical Analysis

For each of the eight cows, a regression equation for a line that related the percentage of

positive cells for each cell marker to day was applied between d -13 to 0 and between d 0.5 to

13. A t test statistic was used to test whether the mean of these slopes differed from 0.

RESULTS

CD3$^+$ Cells (Total T Cells)

Thirteen days before parturition, $47.4 \pm 2.1\%$ (mean $\pm$ SEM) of PBMC expressed CD3

antigen (Figure 1). This percentage gradually decreased and reached a nadir on d 1 after

calving ($35.1 \pm 5.2\%$). From d 0.5 until d 13 postpartum, there was a gradual increase in the

percentage of CD3$^+$ T cells to $49.8 \pm 2.1\%$ (d 10). The slopes of the linear regression lines

from d -13 to 0 and d 0.5 to 13 were -0.43 ± 0.12 and 1.20 ± 0.24, respectively. The

probabilities that these slopes equaled 0 were 0.013 and 0.002, respectively, indicating that

both the decrease in the percentage of CD3$^+$ T cells before parturition and the subsequent

increase after parturition were statistically significant.

CD4$^+$ Cells (T-Helper Cells)

Thirteen days before parturition, $30.2 \pm 1.8\%$ of PBMC expressed CD4 antigen (Figure 1). The percentage decreased in a manner similar to that of the CD3$^+$ T cell population, reached a nadir on d 1 after calving ($22.5 \pm 3.6\%$), and gradually recovered to $30.3 \pm 1.7\%$ (d 6). The slopes of the linear regression lines from d -13 to 0 and d 0.5 to 13 were -0.28 ± 0.06 and 0.70 ± 0.13, respectively. The probabilities that these slopes equaled 0 were 0.002 and 0.001, respectively. Just as with CD3$^+$ T cells, percentages of CD4$^+$ T cells decreased significantly before parturition and increased significantly after parturition.

CD8$^+$ Cells (T-Cytotoxic/Suppressor cells)

The percentage of CD8$^+$ T cells decreased before parturition and increased after parturition (Figure 2). Although the slopes of the linear regression lines from d -13 to 0 and d 0.5 to 13 were -0.12 ± 0.05 and 0.24 ± 0.13, respectively, the probabilities that these slopes equaled 0 were 0.054 and 0.102, respectively, indicating that these changes were not statistically significant.

$\gamma\delta$-T Cells

As with the aforementioned three T-cell subsets, $\gamma\delta$-T cell populations decreased before parturition and increased after parturition (Figure 2). The value on d -13 was $7.3 \pm 0.9\%$ with a nadir on d 0.5 ($5.4 \pm 0.6\%$). By d 10, the percentage of cells had recovered ($7.9 \pm 0.9\%$). The slope of the linear regression lines from days -13 to 0 and days 0.5 to 13 were -0.11 ± 0.05 and 0.19 ± 0.05, respectively. The probabilities that these slopes equaled 0 were 0.049 and 0.005, respectively, thus these changes were statistically significant.

Interleukin-2 receptor α chain positive cells

Interleukin-2 receptor α chain positive cells are thought to be an index of "activated" cells. There was a wide day-to-day variation in interleukin-2 receptor positive cells (minimum 18.61 ± 3.31% on day 1; maximum 23.54 ± 2.01% on day -13) and no significant patterns were observed.

CD4/CD8 ratio

CD4/CD8 ratios were variable with no definite pattern during the sampling period (minimum 2.47 ± 0.33 on d 8; maximum 3.37 ± 0.65 on d -10).

Total Leukocyte Count

No significant changes were observed during the sampling period, though the leukocyte count tended to be increased on d -2 (7325 ± 799/mm³). The leukocyte count on d -13 (6540 ± 469/ mm³) was similar to that observed on d 10 (6219 ± 623/ mm³) (Figure 3).

Mononuclear Cell Count

There were large day-to-day variations in PBMC (minimum 2675 ± 377/mm³ on d1; maximum 3873 ± 3873 ± 531/mm³ on d -4), and no significant patterns were observed (Figure 3).

DISCUSSION

In an earlier study (6), in which cows were sampled at weekly intervals, changes in percentage of CD8 positive cells were not detected, and CD4 positive cells in PBMC exhibited an increase following parturition. When the frequency of sampling around parturition was increased, we were able to demonstrate that the percentages of CD3, CD4, and γδ-T cell

receptor positive cells, declined significantly before parturition and reached a nadir at parturition. The nadirs of each subpopulation represented about a 25% decline in the proportion of each subset in the circulation. These percentages then gradually returned to prepartum values during the first 2 wk of lactation.

The CD4/CD8 ratio was variable but remained above 2 as seen in the previous study (6), suggesting that CD4/CD8 ratio is of little value as an index of periparturient immunosuppression.

We suspect that the impairment of immune cell function in periparturient dairy cows (10, 11) is associated with decreasing T-cell subset populations because the two events occur around the same time during the parturient period. Because we did not measure immune function in this study, we cannot establish a relationship between the decline of T-cell percentages and diminished immune cell function in the same cows. Shafer-Weaver et al. (13) showed a correlation between decreased T-cell subsets (CD2, CD4, CD8, and CD5 positive cells) and diminished lymphocyte proliferation, cytokine (interleukin-2 and interferon-γ) activity, and cytotoxicity stimulated by interleukin-2 in postpartum cows in comparison with midlactation cows. Our data support their findings and extend them by demonstrating that the important changes in T-cell subsets (decline before parturition, reaching nadir at parturition, and increase after parturition) are seen during the 2 wk before and after calving. T-Helper cells activate both humoral and cell-mediated immune responses as well as the antigen nonspecific phagocytic cells (neutrophils, macrophages and natural killer cells) and recruit neutrophils to sites of inflammation by producing various cytokines and chemotactic factors. Although the exact function of γδ-T cells is still unknown, these cells are thought to be responsible for defense against intracellular microbial infections and may be a source of various cytokines which activate γδ-T cells themselves and other neighboring immune cells in an autocrine and paracrine manner (5). Although not statistically significant, the CD8 positive cell population was also decreased at calving. These cells play a major role in protection and

recovery from viral, certain bacterial, and parasitic infections, and also contribute to tumor regression after activation by T-Helper cells. We suspect that a decline in CD4 , CD8, and $\gamma\delta$-T cell receptor positive cells as parturition approaches may be linked to the immunosuppression observed in periparturient dairy cows because the two events occur at around the same time.

CONCLUSION

Numerous studies have demonstrated a decline in immune cell function during the periparturient period. In this study we have demonstrated that populations of T-cells, especially T-Helper ($CD4^+$) and $\gamma\delta$-T cells ($N12^+$) decline during the periparturient period as well, in a pattern very similar to that reported for loss of immune cell function. Whether the cell population changes are the cause or the effect of periparturient immune suppression remains unknown.

ACKNOWLEDGMENTS

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TABLE 1. Primary antibodies used to identify mononuclear cells.[1]

Antigen	MAb clone (murine)	Isotype	Working Ab concentration[2] (μg/ml)	Specificity
CD3	MM1A	IgG_1	7	Total-T cell
CD4	GC50A1	IgM	14	T-Helper cell
CD8	CACT80C	IgG_1	21	T-Cytotoxic/ Suppressor cell
N12	CACT61A	IgM	14	$\gamma\delta$-T cell receptor
CD25	CACT108A	IgG_{2a}	28	Interleukin-2 receptor α chain

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

2 Monoclonal antibody diluted in PBS with 1% fetal bovine serum.

Figure 1. Percentage of peripheral blood mononuclear cells (PBMC) from periparturient Jersey cows (X ± SEM; n = 8) that were positive for CD3 (Total T cell; ☐) or CD4 (T-Helper cell; ●) antigens.
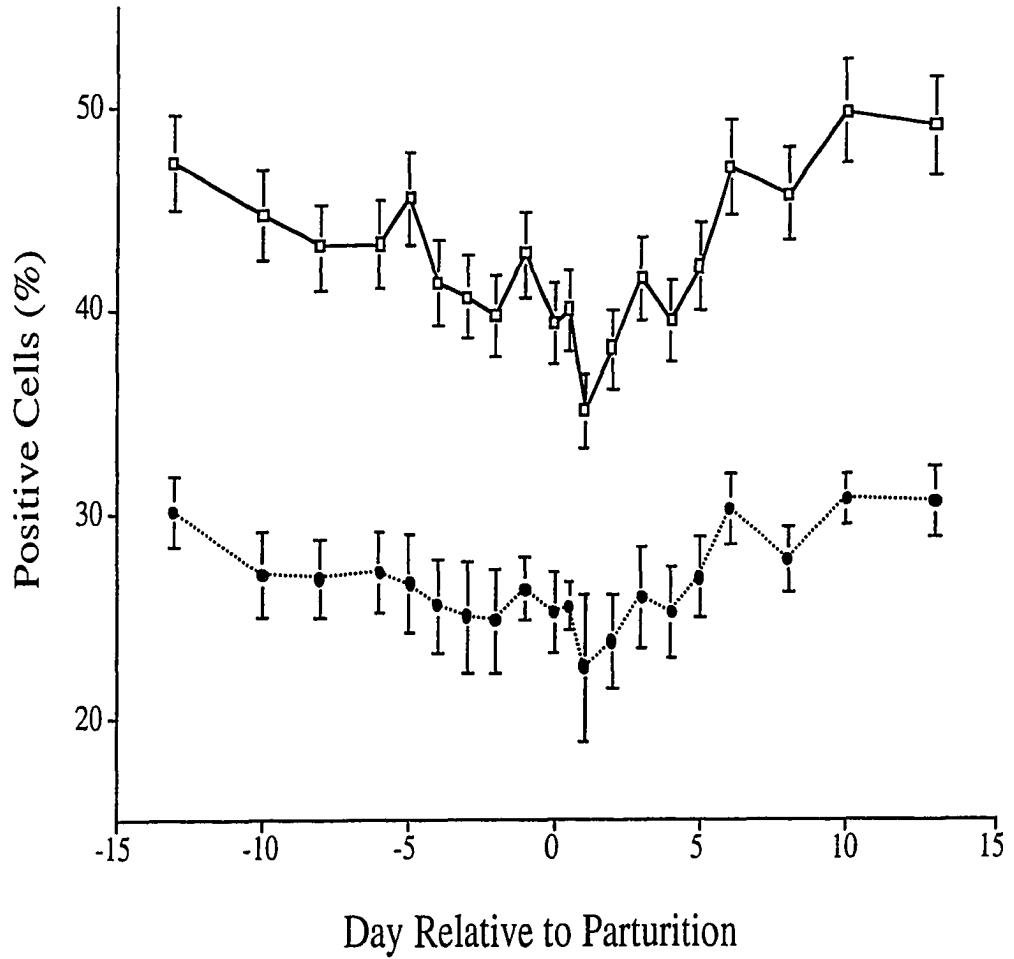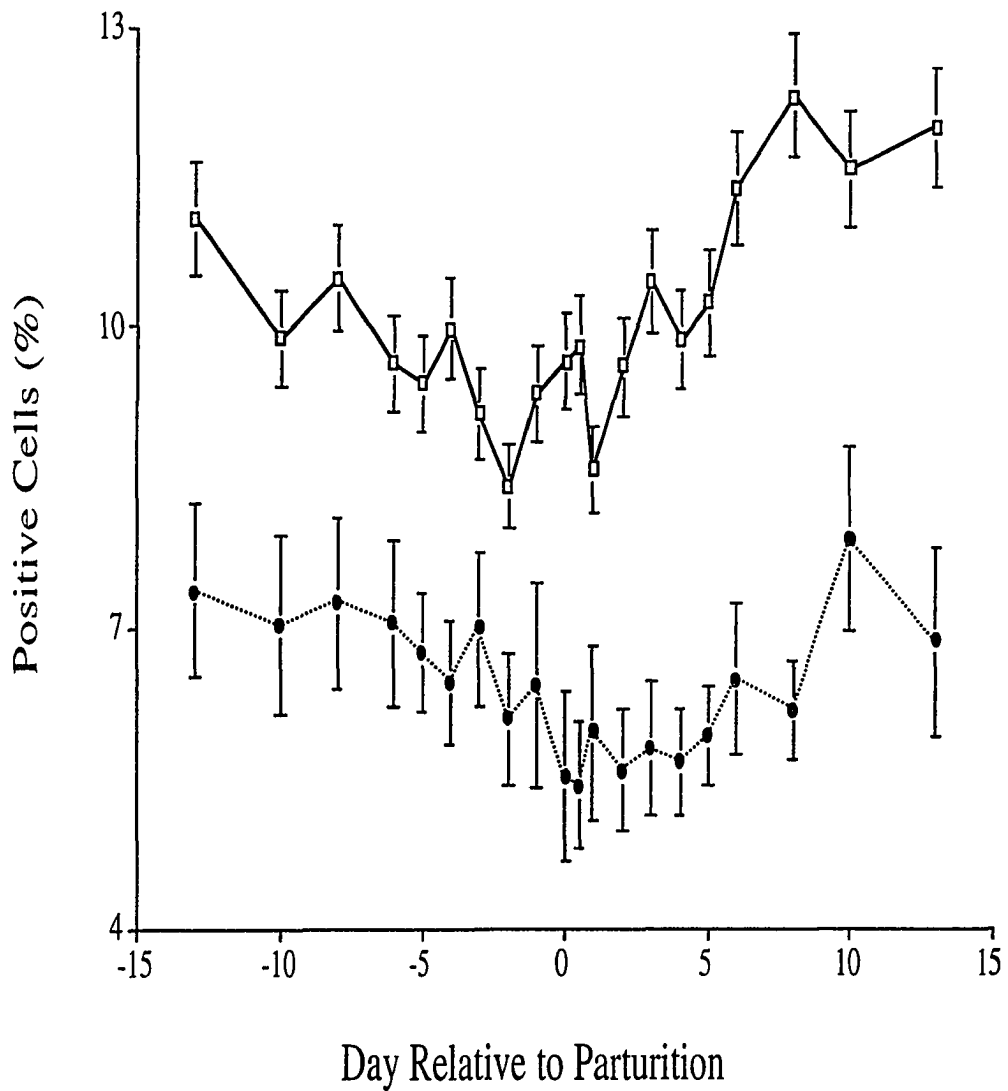
Figure 2. Percentage of peripheral blood mononuclear cells (PBMC) from periparturient Jersey cows (X ± SEM, n = 8) that were positive for CD8 (T-Cytotoxic/Suppressor cell; □) or N12 (γδ-T cell; ●) antigens.
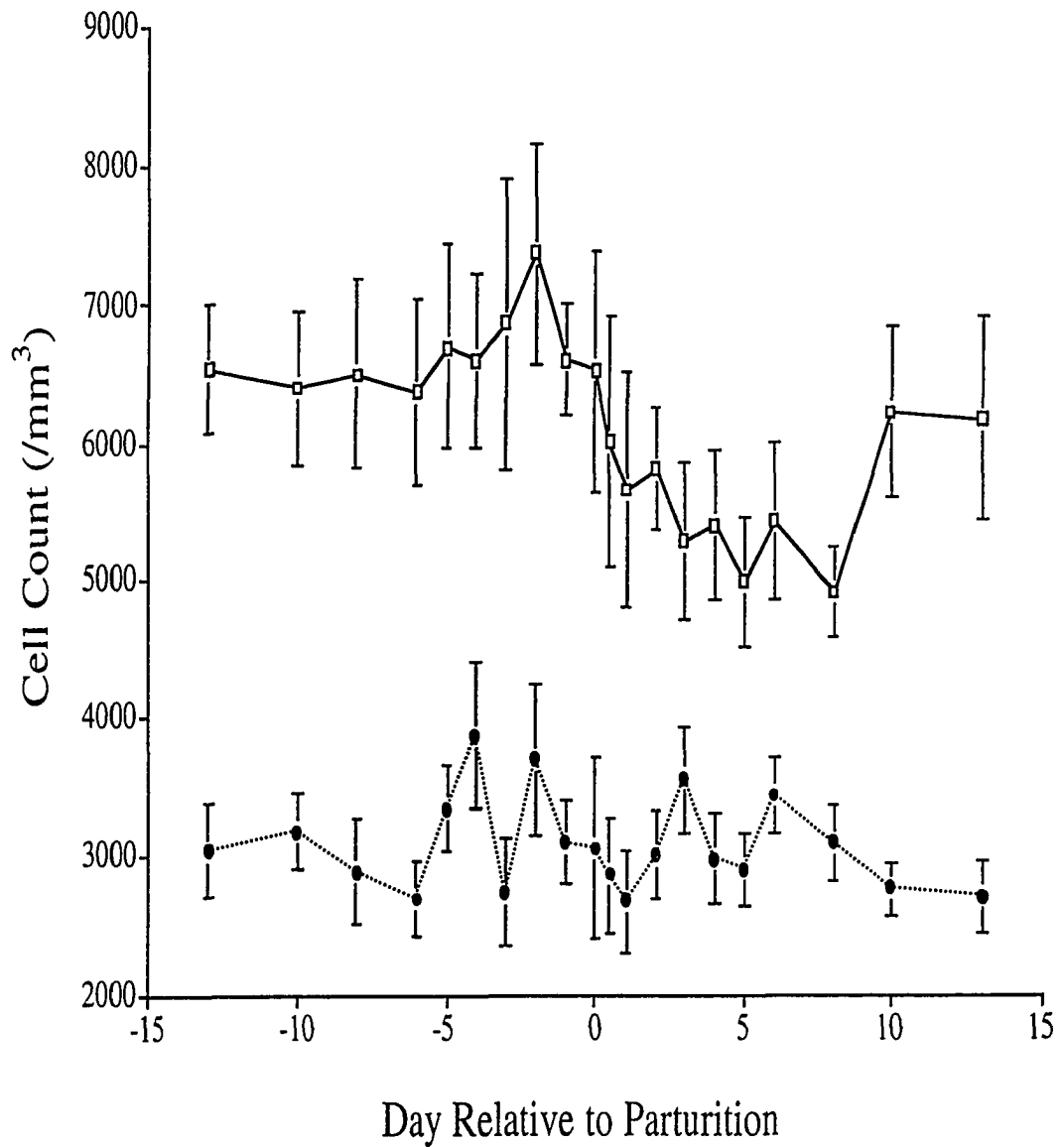
Figure 3. Cell counts of total leukocytes (□) and mononuclear cells (●) (X ± SEM; n = 8).

EFFECTS OF THE PRESENCE OF THE MAMMARY GLAND ON PHENOTYPE OF PERIPHERAL BLOOD MONONUCLEAR CELLS IN PERIPARTURIENT DAIRY COWS

A paper to be submitted to the Journal of Dairy Science

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ABSTRACT

There is a high incidence of disease, such as mastitis, in cows during the periparturient period. We previously established a significant decline in several T-cell subsets and diminished lymphocyte and neutrophil function in periparturient dairy cows. The reason for this periparturient immunosuppression is unknown. We hypothesized that milk production may be an important immunosuppressive factor. The endocrine changes associated with parturition and onset of lactation, and metabolic stress associated with the onset of milk production are suspected to be factors contributing to the periparturient immunosuppression. To attempt to determine whether the changes in mononuclear cell phenotype observed around parturition around parturition in dairy cows are due to the parturition process or the stress

associated with onset of lactation, we used 10 mastectomized and 8 intact multiparous Jersey

cows. Flow cytometry permitted phenotype analysis of peripheral blood mononuclear cells

(**PBMC**) using monoclonal antibodies against T-cell subsets, B-cells, and monocytes. Blood

samples were taken from -4 to 4 wk after parturition. In intact cows, all T cell subset

populations (CD3, CD4, CD8 and γδ-T cell receptor positive cells) showed a significant

declining pattern toward parturition, while the monocyte percentage increased significantly at

parturition in intact cows. These changes were significantly different from mastectomized

cows except for the CD8 positive cells. Mastectomy eliminated almost all changes in

leukocyte subsets seen at the time of parturition.

(**Key words:** immunosuppression, periparturient, peripheral blood mononuclear

cells, phenotype analysis, T cell subsets)

Abbreviation key: ACD = acid citrate dextrose, **IL-2r** = interleukin-2 receptor, **MAb** =

monoclonal antibody, **MHC-II** = major histocompatibility complex class II, **WBC** =

leukocytes, **PBMC** = peripheral blood mononuclear cells, **ANOVA** = analyses of variance

INTRODUCTION

Parturition is an important and necessary process for dairy cows to produce milk. The high

incidence of disease at around parturition causes significant damage to cows and the dairy

industry; the results of which are the subsequent decrease in milk production and the decline in

the general health of herd. It is already known this increased susceptibility to infectious

diseases, especially mastitis, is due to the impaired neutrophil and lymphocyte functions in

periparturient dairy cows (4, 16, 17, 19). In order for the immune system to work

effectively, the appropriate cell population should migrate to the appropriate location. Since

the blood stream is the vehicle for delivering immune cells to the sites of egress into tissues as

needed, we suspected that this immunological dysfunction is related to the population of circulating leukocytes (WBC) subsets available to combat new infections.

WBC are classified as shown in Figure 1. These cells have distinct and common functions, as well as unique cell surface molecules which help us to analyze these cell population as shown in Figure 2. Immune cell population phenotypic analysis revealed a relationship between an inappropriate balance in immune cell subsets and lowered immune function as seen in HIV-infected patients who show decreased populations of CD4 positive cells and immunodeficiency (21). An association between function and cell population of PBMC has been reported in glucocorticoid immune suppression models (24). Using 8 multiparous Jersey cows during the periparturient period (-2 to 2 wk after parturition), we found that there is a significant decline in several T cell subset populations before parturition and recovery after parturition showing a nadir at calving (20). This change in circulating leukocyte subpopulations was seen at time where previous studies with periparturient dairy cows showed nadir of lymphocyte and neutrophil function around parturition (4, 18, 19). It is suspected that the decline of T cell subset populations may be a contributing factor for periparturient immunosuppression. A correlation between decreased T cell subsets and diminished lymphocyte functions in postpartum cows in comparison with mid-lactating cows has been demonstrated (27). However, the reason for this decline in circulating T cell population` and decreased immune cell function in periparturient dairy cows is still unknown. Cows are exposed to the tremendous changes during the periparturient period due to induction of parturition and onset of lactation. These changes are caused by or resulted in the severe physiological changes such as hormonal and nutritional changes. We hypothesized that colostrum production may be an important immunosuppressive factor. In order to test this hypothesis, we used 10 mastectomized and 8 intact multiparous Jersey cows to see the effect of mammogenesis and lactogenesis on the immune system. In this paper we focus on the effects of lactation and the mammary gland on the phenotype, and the companion paper

describes the function of PBMC. We analyzed all PBMC subsets using a bovine specific

monoclonal antibody (**MAb**) against markers shown in Fig. 2 except for the NK cell

population which has no MAb available for bovine cells at present.

MATERIALS AND METHODS

Animals

Eighteen multiparous Jersey cows between 5 and 8 years of age were evaluated in this

study. Ten cows were mastectomized during the 3rd-5th month of gestation and allowed to

heal. The cows calved between February 1996 and January 1997. Eight intact cows, due to

calve around the same time as each mastectomized cow, were chosen as controls so that

reagents used on mastectomized cow PBMC could also be used to assess PBMC analysis of

intact cows, thus reducing the effects of time and season. The cows were managed as

similarly as possible and cared for by the same caretakers for the duration of the study. Cattle

were housed in a free-stall barn until 2 to 3 d before calving, when they were brought into

maternity pens. They were fed alfalfa-corn silage based diets prior to and after calving with a

dietary high cation-anion difference $[(Na^+ + K^+) - (Cl^- + S^{2-}) = +400$ meq/kg]. This type of

diet is still fed on many dairy farms and is known to increase the probability of metabolic

stress such as milk fever in cows.

For phenotype analysis and leukocyte counts, blood samples were taken by jugular

venipuncture into tubes containing the anticoagulants acid citrate dextrose (**ACD**) and

ethylenediamine tetraacetic acid respectively. Samples were taken from 4 wks before the

expected calving date to 4 wks after calving. The bleeding frequency was twice a week for

wks of -4,-3, 3, and 4; three times a week during wks of -2 and 2; daily during wks of -1 and 1. On the day of calving, samples were taken at 0, 12 and 24 hrs after calving.

Enumeration of peripheral blood mononuclear cells

Total WBC counts were determined with whole blood collected in tubes containing ethylenediamine tetraacetic acid using an electronic cell counter (CellTrack, Angel Engineering Corp, Trumbull, CT). Differential cell counts were done using flow cytometry. PBMC and PMN were identified based on the forward and side scatter characteristic on dot plots (2). The total number of PBMC per cubic millimeter of whole blood was calculated from these data.

Cell preparation and antibody binding

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

Antibodies

Monoclonal antibodies were used to identify cell surface markers, which allowed us to categorize the PBMC fraction of the whole blood. The primary antibodies used are all specifically reactive to bovine markers (1, 14) and descriptions of working solutions are presented in Table 1. The secondary antibodies used were FITC-conjugated goat anti-mouse IgG (H+L) F(ab)$_2$ (Caltag Laboratories, San Francisco, CA) or PE-conjugated goat anti-mouse IgM (H+L) F(ab)$_2$ (Southern Biotechnology Associates, Inc., Birmingham, AL) which were diluted 1:100 or 1:500, respectively, in PBS with 1% fetal bovine serum for use as a working solution.

Flow cytometric analysis

A FACScan flow cytometer (Becton Dickinson, San Jose, CA) was used to acquire data on fluorescent scattering associated with the presence of antibodies on cell surface antigens (CellQuest™ software; Becton Dickinson). Data from 5000 events/sample were acquired. For all analyses (CellQuest software™; Becton Dickinson), mononuclear cells were gated out from granulocyte populations based on their forward and side scatter characteristics on density plots (3).

Statistical analysis

To investigate the impact of mastectomy on relevant variables, we performed split-plot repeated measure analyses of variance (**ANOVA**), followed by a simple effect analyses (Duncan's multiple comparisons). The non-repeated measure factor was treatment (intact vs. mastectomy) and the replication factor, cow. The repeated measure factor was time (days

relative to parturition). (Because of some missing data, the ANOVA data were analyzed using the general linear model partial sums of squares procedure.)

RESULTS

The changes in the marker-positive cell populations are shown in Figures 3 - 10. The results of statistical analysis are shown in Table 2.

General observations

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

T cell populations

As the low probability of treatment*time shows, CD3, CD4, $\gamma\delta$-T cell receptor positive cells showed significantly different patterns of change during the sampling period, between intact and mastectomized cows. Whereas CD8 positive cells did not show any treatment*time effect due to the declining pattern in both groups, although this decline was minor in mastectomized cows (Figures 3 - 6). That is, CD3, CD4, CD8, and $\gamma\delta$-T cell receptor positive cells in intact cows showed declined from a d -27 value of 51.9 $\pm$ 1.9 (mean $\pm$ SEM), 29.8 $\pm$ 1.3, 12.3 $\pm$ 2.4, and 7.2 $\pm$ 1.0 % to nadir at parturition with 40.1 $\pm$ 2.2 (d -1), 24.8 $\pm$

2.3 (d 0.5), 8.6 ± 1.0 (d -1) and 4.4 ± 0.4 (d 0.5) respectively. These values increased after parturition and reached preparturient values except the γδ-T cell receptor positive cells which did not show complete recovery. Conversely, these marker positive cells in mastectomized cows showed day-to-day variation throughout the sampling period for CD3 (52.9 - 49.5), CD4 (31.5 - 28.5), CD8 (13.9 - 11.9), and γδ-T cell receptor (9.0 - 7.2) positive cells, respectively. Since there was a sustained low value of γδ-T cell receptor positive cells in intact cows after parturition, this population also showed a significant treatment effect.

CD4/CD8 ratio

CD4/CD8 ratios were variable between 3.0 to 3.6 in intact cows and between 2.3 and 2.9 in mastectomized cows with no definite pattern during the sampling period, and the difference between both groups was not significant.

Interleukin-2 receptor α chain positive cells

There was a wide day-to-day variation in interleukin-2 receptor α chain positive cells in both groups (Figure 7); therefore there were no treatment, time, or treatment*time effects in the overall analysis. However, there was a declining pattern in this population in intact cows from d -8 to d 0.5, reaching a nadir on d 0. The difference in values on d 0 between groups was not significant (p = 0.094).

Major histocompatibility complex class II antigen (MHC-II) positive cells

MHC-II positive cells showed a higher value before parturition and they remained low after calving in intact cows (Figure 8); whereas mastectomized cows showed day-to-day variation

throughout the sampling period. There was a marked difference between both groups from the beginning of sampling until parturition. Intact cows had more MHC-II positive cells on d 27 with 32.4 ± 2.0 % compared to 25.9 ± 2.2 % in mastectomized cows. Although there was a decline in MHC-II positive cells after parturition in intact cows, the values remained higher than mastectomized cows showing significant treatment and treatment*time effects.

B cells and Monocytes

The B cell population showed no definite change during the sampling period in both groups (Figure 9). In contrast, monocytes showed a distinct increase at parturition (Figure 10). Monocyte population increased in intact cows as parturition approached reading a zenith on d 0
(29.0 ± 2.4 %) and declined; whereas mastectomized cows showed a slight decline in monocytes toward parturition and almost no change after parturition. The difference in monocyte population between both groups was significant (Table 2).

Enumeration of leukocytes

As Figure 11 shows, there was an increase in total WBC prior to calving and a decrease after parturition in both groups. PBMC showed a slight decrease at parturition in both groups (Figure 12). Intact cows continued to decrease after calving but mastectomized cows showed recovery until the end of sampling. Statistical analysis showed time effects in both total WBC and PBMC (p=0.095) and treatment effect in PBMC (p=0.055).

DISCUSSION

This study demonstrated that significant changes in PBMC subset populations during the

periparturient period are affected by the presence of the mammary gland, thus mammogenesis

and

colostrogenesis, rather than the sole process of parturition.

Intact cows showed significant declines in all T cell subset populations examined as

parturition approached. These cells returned to preparturient proportions after parturition with

the one exception of γδ-T cells which remained low through the end of the sampling period.

Marked changes were seen during -2 to 2 wks after parturition, similar to our previous study

(20). A nadir of proportions of these subsets was seen from d -1 to 1 in the former study as

well as in this study; whereas the monocyte percentage showed an opposite trend with an

increase toward parturition and a decrease after parturition. This tells us that the decline in T

cell population is counter balanced by monocyte population. Changes in T cell and monocyte

percentages are similar to the reports on postpartum cows in comparison with mid-lactating

cows (27, 28) . These changes in time were completely different from those of mastectomized

cows except CD8 positive cells which showed no treatment*time interaction; therefore

mastectomy eliminates almost all changes in leukocyte subsets seen at the time of parturition.

Morphological change in the mammary gland starts from about 2 wks before parturition

(30). Colostrogenesis requires nutrition and complex hormonal change (13). Blood flow into

the mammary gland and an influx of leukocytes increase during this period for milk

production (11, 22). Colostrum contains many leukocytes consisting of monocytes,

lymphocytes and neutrophils in this order (23). Changes in the proportion of immune cell

populations of PBMC seen only in intact cows may be due to the effect of nutritional and

hormonal changes associated with lactation. In fact, all intact cows developed milk fever and

3 out of 8 cows had ketosis as well as displaced abomasum, but none of the mastectomized

cows developed diseases during sampling period. This observation proves how significantly colostrum production affects the physiology of cows. One can argue that a milk fever inducing diet (dietary high cation-anion difference) may have caused or enhanced these changes rather than colostrogenesis. We already studied the phenotype of PBMC in intact cows which did not develop milk fever (20). The comparison between current and previous data showed there is no significant difference between milk fever and non-milk fever cows except in the number of $\gamma\delta$-T cell receptor positive cells which remained low after parturition without recovery. Other researchers also showed there was no significant effect of health condition especially metabolic disease on any subset of PBMC in periparturient dairy cows (15). Furthermore, we found that hypocalcemia or the development of milk fever did not exacerbate the immune cell dysfunction in periparturient dairy cows (17). Milk fever may have a certain effect on immunity since milk fever cows have a higher tendency to have mastitis and other diseases (7), but our studies were unable to show any significant effect on the immune system. What about other nutritional effects? Periparturient dairy cows are exposed to significant changes in nutritional state (10, 12), but we don't know how many of these changes resulted from milk production or the terminal process of gestation. We have been studying the change in vitamins and minerals with the same cows used in this study. Those studies may give us further information whether nutrition affects on the change in immune cell population, thus immune function.

Influx of leukocytes into the mammary gland can be the reason for decreased T cell subsets in circulation. However, it is unlikely that the significant decrease in circulating T cell subsets are due to the preferential influx of T cells into the mammary gland. It is reported that a similar decline in T cell subsets and an increase in monocytes were observed in the mammary gland concomitantly with PBMC in postpartum cows (within 3 d after calving) compared to mid-lactation cows (27). Changes in the PBMC reflect the change in mammary gland cell population. Park et al. showed a significant decline in CD2, CD4, and CD8 positive cells

within 48 h after parturition compared to other lactating and non-lactating stages in mammary gland secretion (25). Taylor et al. found the lowest percentage of CD4 positive cells in the early lactation compared to later stages of lactation in mammary gland secretion (33). How do these changes occur? One possibility is the increased cell death of T cells, and the other is the T cell redistribution to tissues. Both possibilities related to increase in glucocorticoid level have been reported (3, 5, 8). Indeed, increased cortisol

levels observed at parturition in cattle (9)would seem to be inversely correlated with the changes of marker positive cell populations found in our present study, and this result is consistent with previous findings with dexamethasone (3). However, it is questionable that all the changes in cell percentage observed in our study can be due to the acute rise in glucocorticoids observed in cows at parturition, especially since changes were seen prior to the spike in plasma cortisol. Furthermore, we already know that there was no significant difference in increased plasma cortisol levels between intact and mastectomized cows (unpublished data). We also measured other steroid hormones (estrone, estradiol, and progesterone) which are thought to be responsible for periparturient immunosuppression because of their tremendous change in plasma level in the period of parturition (6, 29). We found that these factors are not likely to be the answer for the changes in immune cell population, either, because they showed similar (progesterone) or higher levels in mastectomized cows in comparison with intact cows (unpublished data). Other lactation related hormones may cause the changes in cell population, but the answer remains unknown.

Higher percentage of MHC-II positive cells in intact cows before parturition is an interesting observation. It agrees with another report (15). As mastectomized cows did not show any change during sampling period, it may be due to the increased synthesis of TNF-α in the mammary gland reported during drying period (31, 32, 34). Increase in TNF-α without infection was speculated to be important for tissue remodeling necessary for colostrogenesis (34). As TNF-α stimulates the expression of MHC-II (26), increased TNF-α

in the mammary gland may have spilled over into the blood stream and stimulated MHC-II expression in intact cows. But this expression did not seem to be correlated to the increase in monocytes. A higher percentage of monocytes without MHC-II expression, i.e., a higher level of non-activated monocytes may be one of the reasons for periparturient immunosuppression seen only in intact cows (see Appendix).

As our companion paper with the same cows used in this experiment (6 cows for each group) shows (see Appendix), the changes in immune cell population seem to be well correlated to the decreased PBMC function seen only in intact cows. This agrees with the study by Shafer-Weaver et al. who showed decreased T cell subsets (CD2, CD4, CD8 and CD5 positive cells) and increases in monocyte population in periparturient cows (within 3 days of parturition) compared to non-lactating cows were correlated to diminished function of lymphocytes with various assays (proliferation, interleukin-2 and interferon-γ activity, and interleukin-2 stimulated cytotoxicity) (27). Our study extends their finding by indicated the effect of the mammary gland on lymphocyte trafficking and immune suppression.

CONCLUSION

This study demonstrated that the presence of the mammary gland and colostrogenesis play an important role in the periparturition immunosuppression as regards T cell population. Significant decreases in T cell populations and an increase in monocyte population are seen only in intact cows, whereas there was almost no change in mastectomized cows. These changes are well correlated to the change in immune function. The reason for these changes may be due to nutrition and hormonal changes associated with milk production.

ACKNOWLEDGMENTS

The authors thank Norman S. Tjelmeland , Creig E. Caruth, Eugene C. Rieks for animal care and Dr. Robert F. Strahan for advice on statistical analysis.

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Table 1. Primary antibodies used to identify mononuclear cells

Antigen	MAb clone (murine)	Isotype	Working solution (MAb diluted in PBS-1% fetal bovine serum)	Specificity
CD3	MM1A	IgG$_1$	7 µl/ml	Total-T cell
CD4	GC50A1	IgM	14 µl /ml	T-helper cell
CD8	CACT80C	IgG$_1$	21 µl /ml	T-cytotoxic cell
N12	CACT61A	IgM	14 µl /ml	γδ-T cell receptor
IL-2r	CACT108A	IgG$_{2a}$	28 µl /ml	Interleukin-2 receptor α chain
MHC-II	TH14B	IgG$_{2a}$	3.5 µl /ml	Class II major histocompatibility complex
B cell	BAQ155A	IgG$_1$	3.5 µl /ml	B cell
Monocyte	BAQ151A	IgG$_1$	3.5 µl /ml	Monocyte

Source of all MAb was VMRD and the concentration of the MAb solution was 1 µg/ml.

Table 2: The results of analysis of variance for each marker positive cells (%) and leukocyte count (/mm^3): P value

Marker	Treatment	Time	Treatment * Time
CD3	0.053	0.0001	0.014
CD4	0.641	0.0002	0.0002
CD8	0.227	0.039	0.943
N12	0.041	0.0001	0.0005
IL-2r	0.585	0.184	0.116
MHC-II	0.015	0.175	0.015
B cell	0.280	0.041	0.507
Monocyte	0.009	0.339	0.082
CD4/CD8	0.246	0.419	0.855
WBC	0.670	0.0001	0.235
PBMC	0.055	0.095	0.842

Immune Cell Classification in PBMC
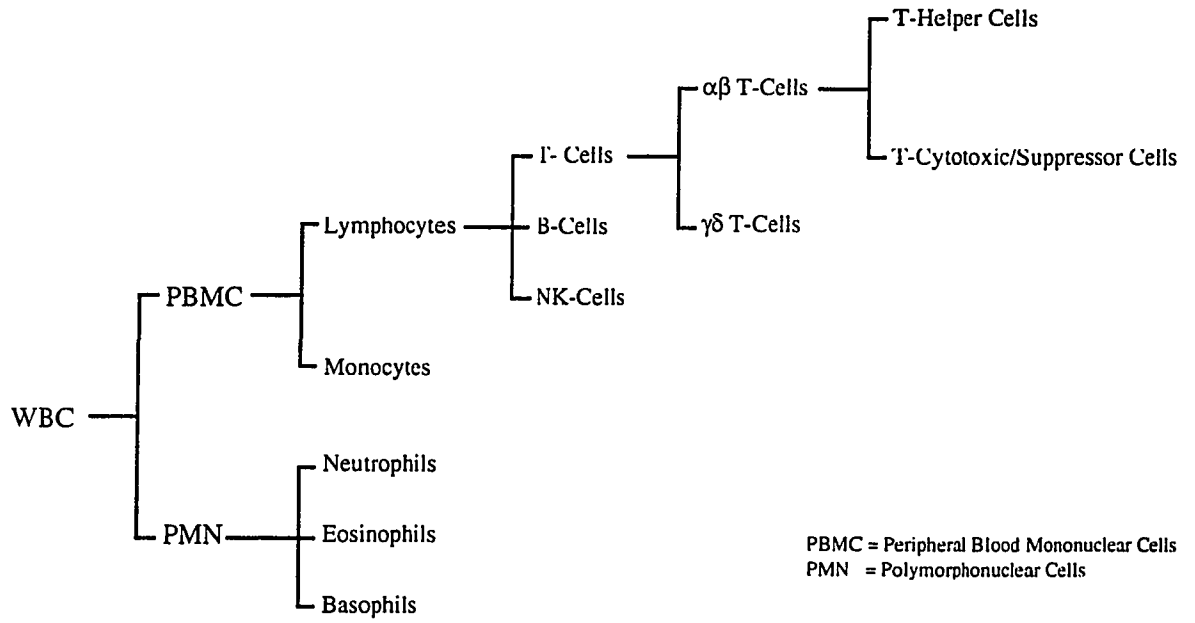
Figure 1. Immune cell classification in PBMC
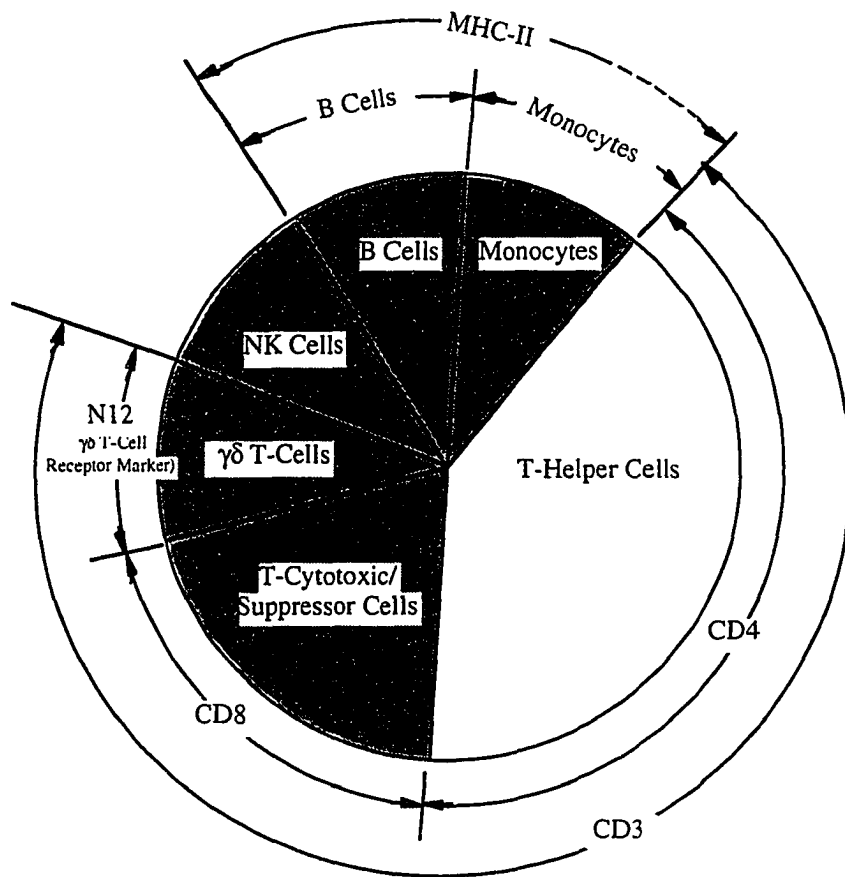
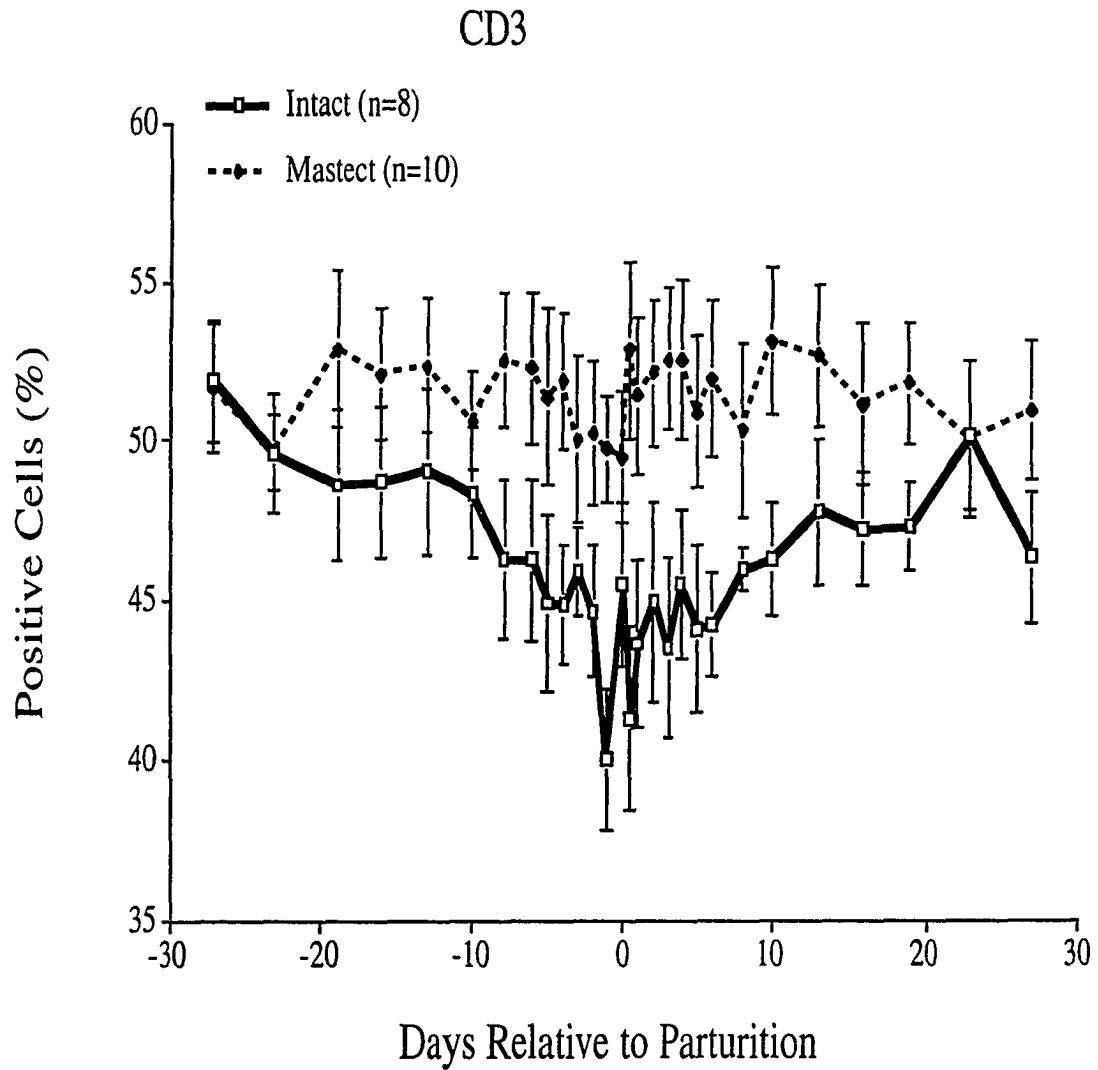Figure 2. Immune Cells in PBMC and Cell Surface Markers Used in This Study

Figure 3 -- Percentage of PBMC positive for the CD3 antigen (Total-T cell) in whole blood from intact and mastectomized cows during periparturient period.
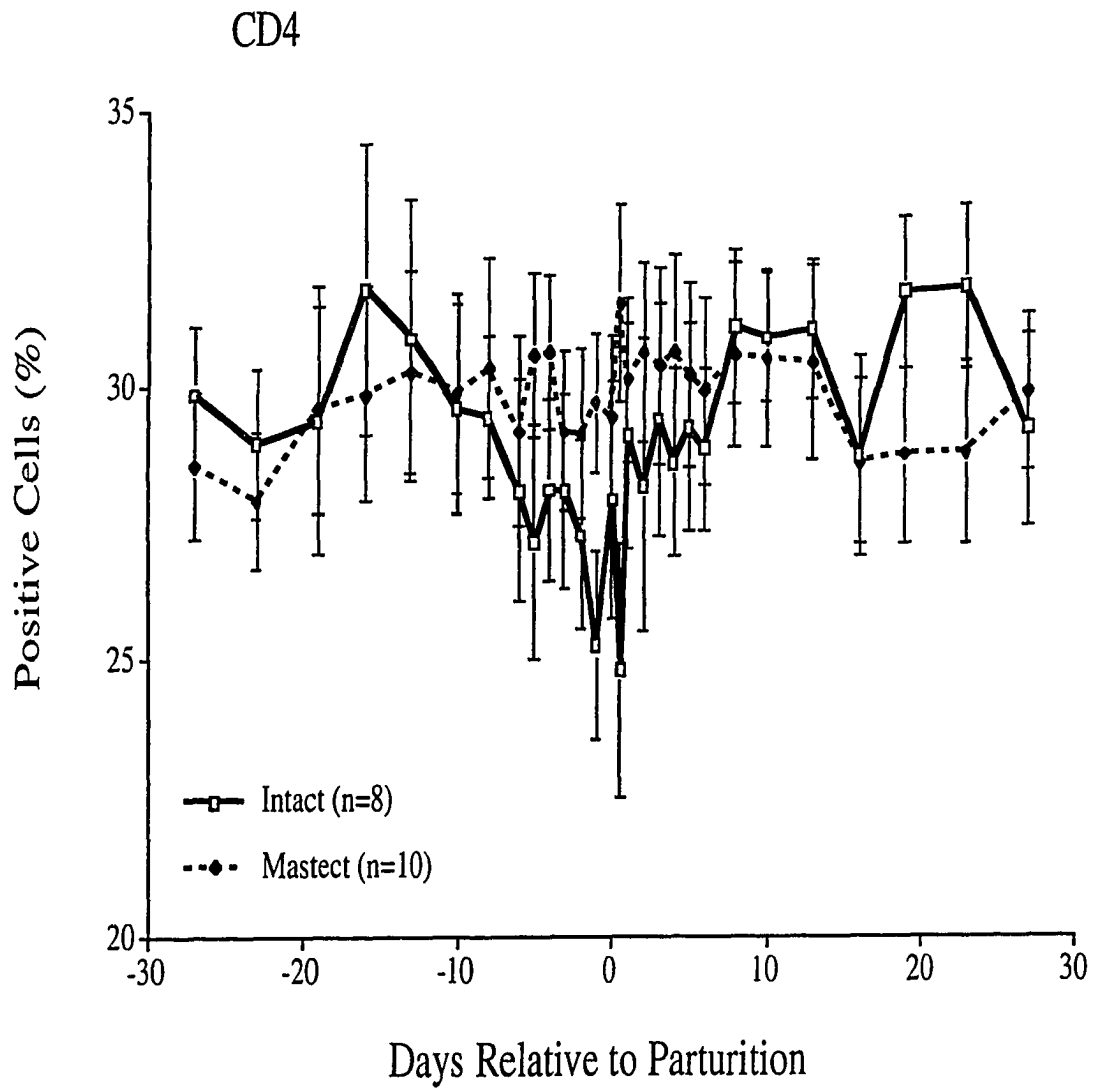
Figure 4 -- Percentage of PBMC positive for the CD4 antigen (T-helper cell) in whole blood from intact and mastectomized cows during periparturient period.
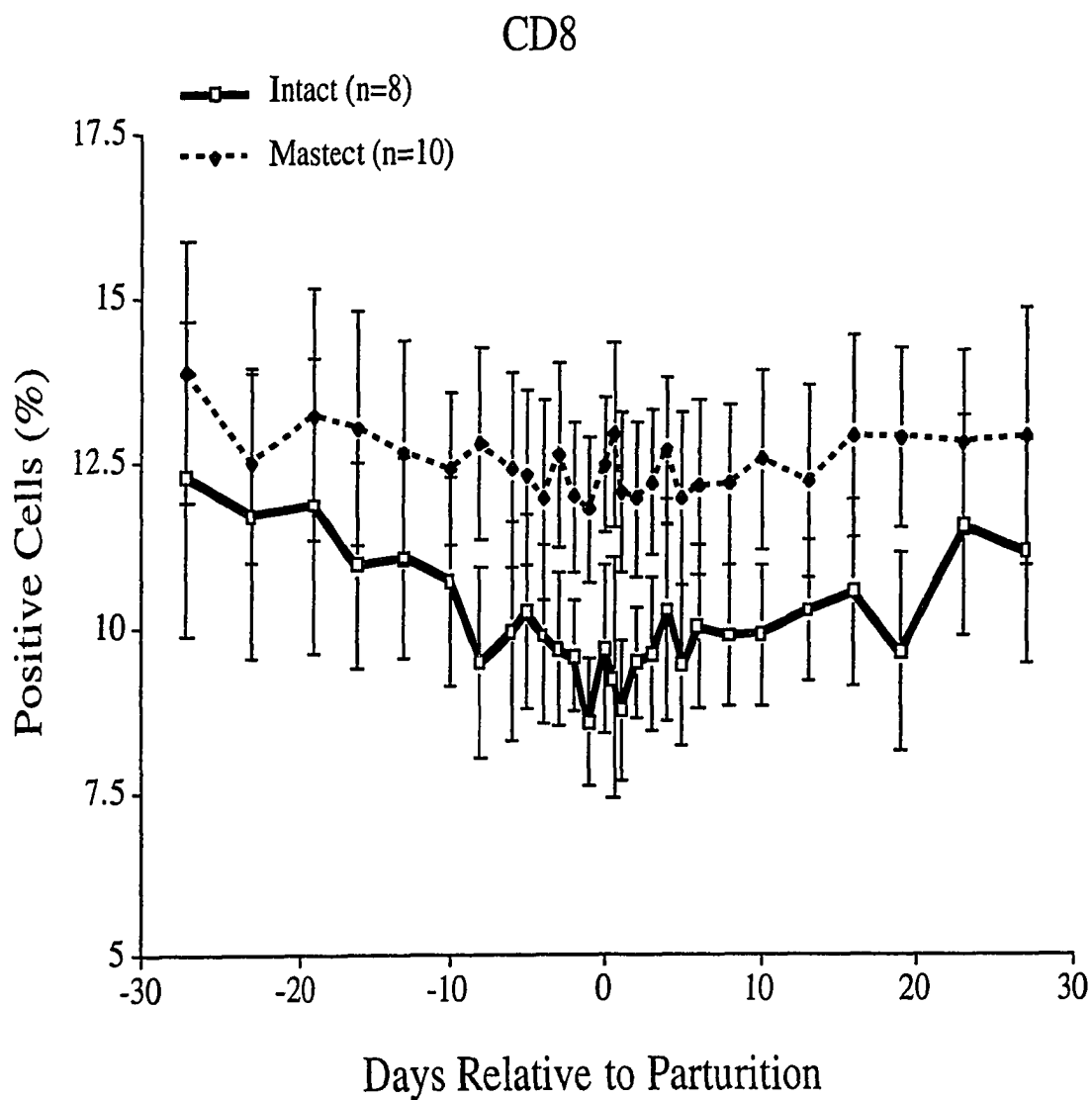
Figure 5 -- Percentage of PBMC positive for the CD8 antigen (T-cytotoxic cell) in whole blood from intact and mastectomized cows during periparturient period.
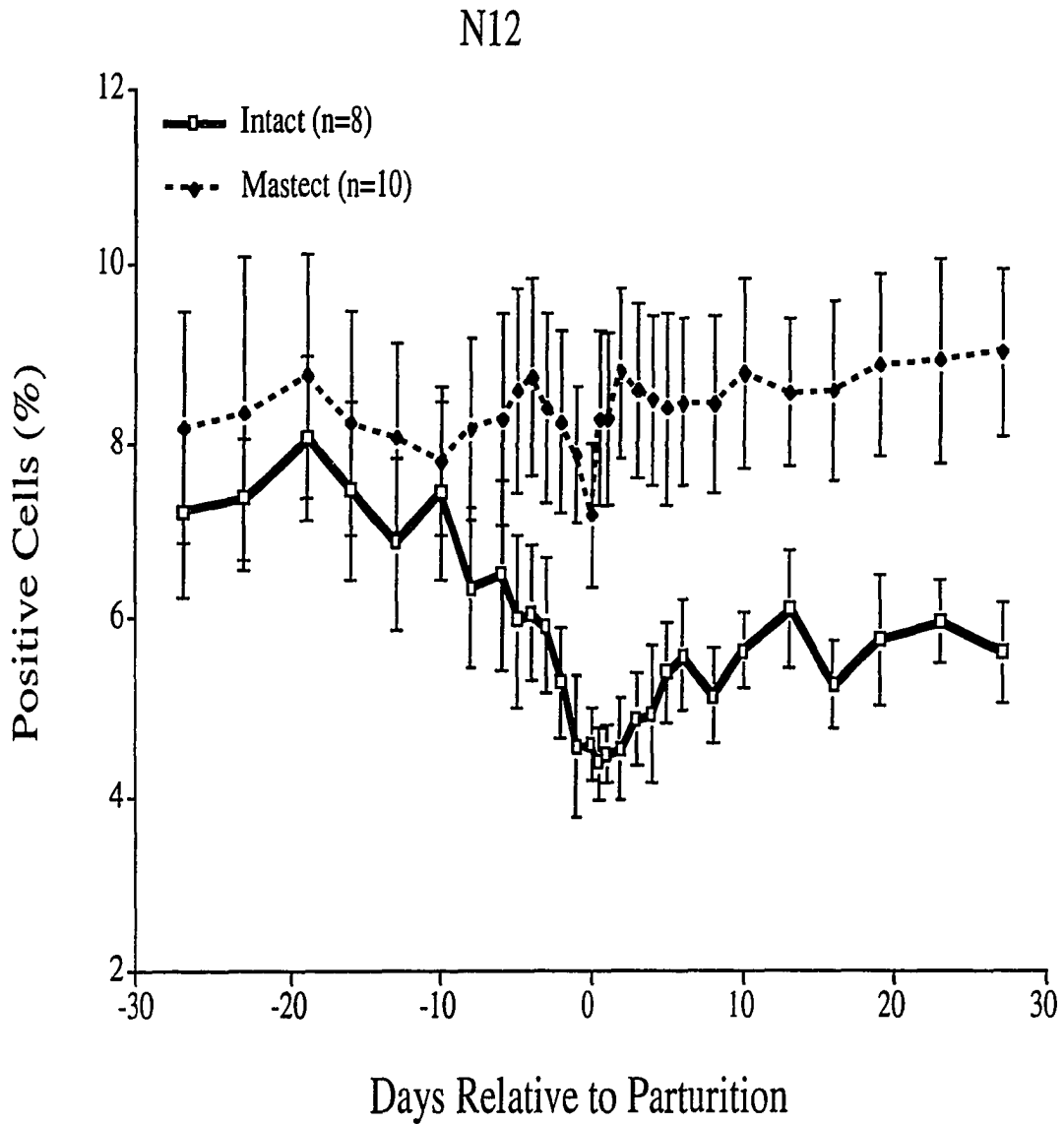
N12

Figure 6 -- Percentage of PBMC positive for the N12 antigen ($\gamma\delta$-T cell) in whole blood from intact and mastectomized cows during periparturient period.
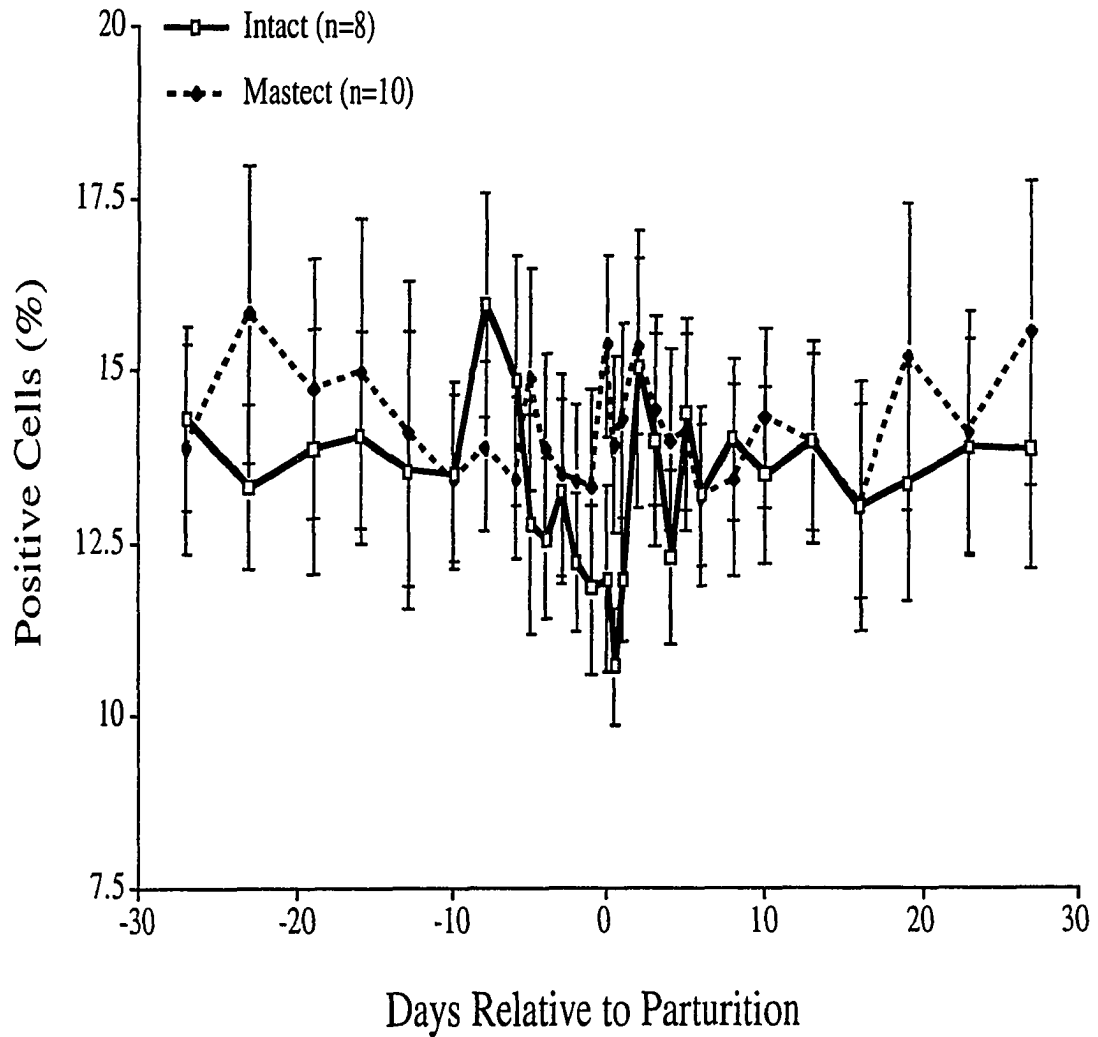
IL-2R

Figure 7 -- Percentage of PBMC positive for the IL-2r antigen (activated lymphocyte) in whole blood from intact and mastectomized cows during periparturient period.
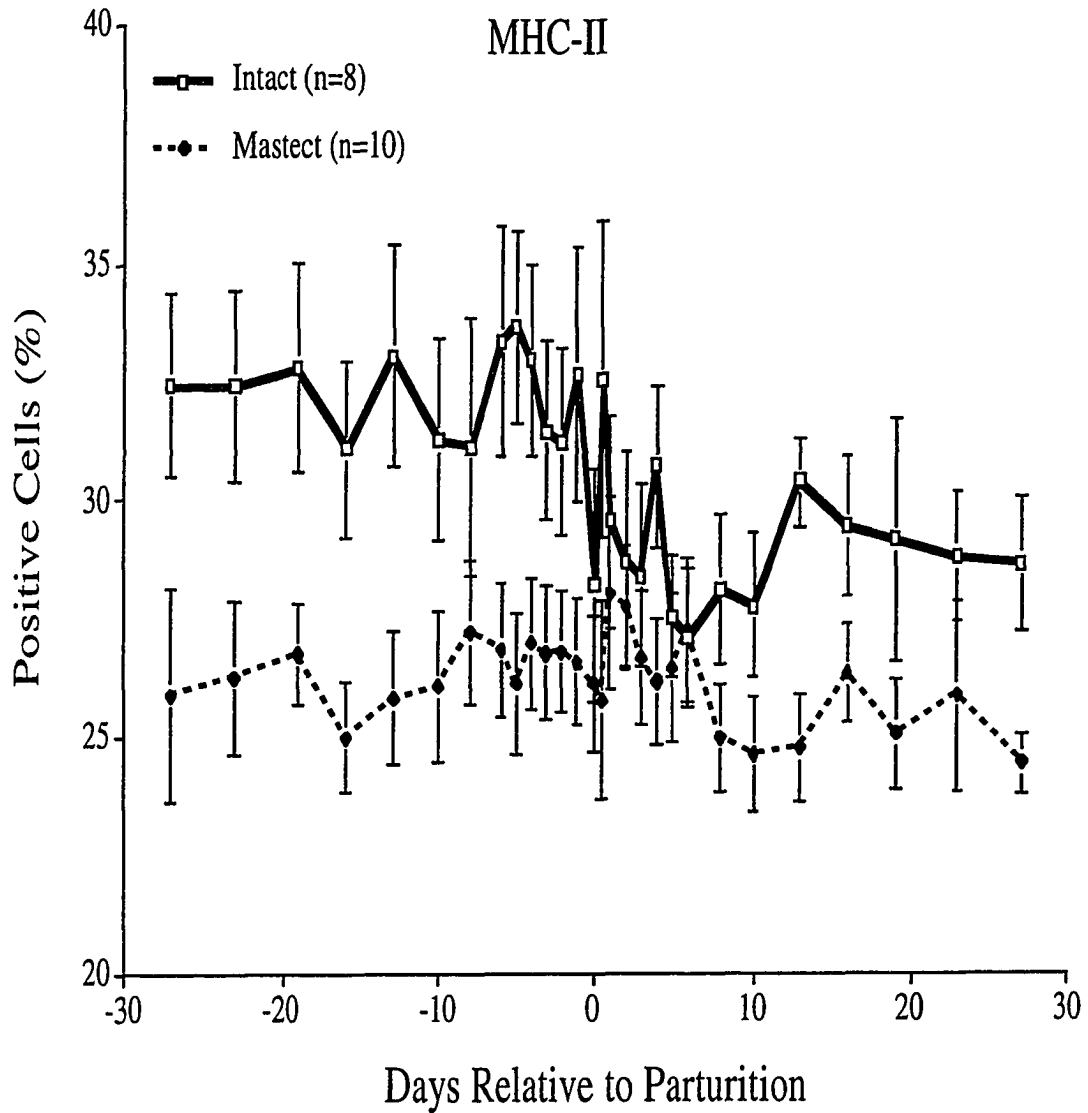
Figure 8 -- Percentage of PBMC positive for the MHC-II antigen (B cell and activated
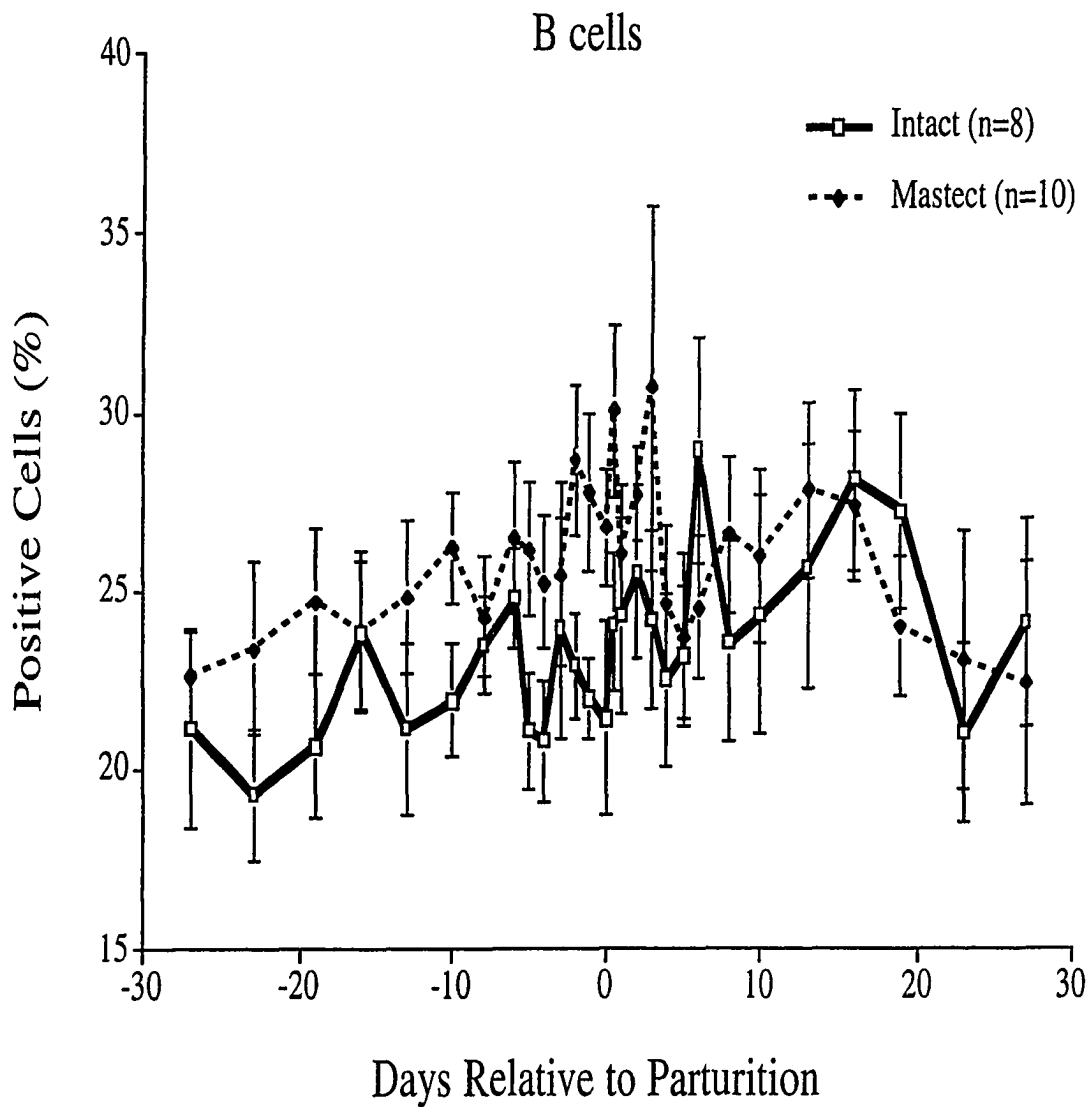monocyte) in whole blood from intact and mastectomized cows during periparturient period.

Figure 9 -- Percentage of PBMC positive for the B cell antigen in whole blood from intact and mastectomized cows during periparturient period.

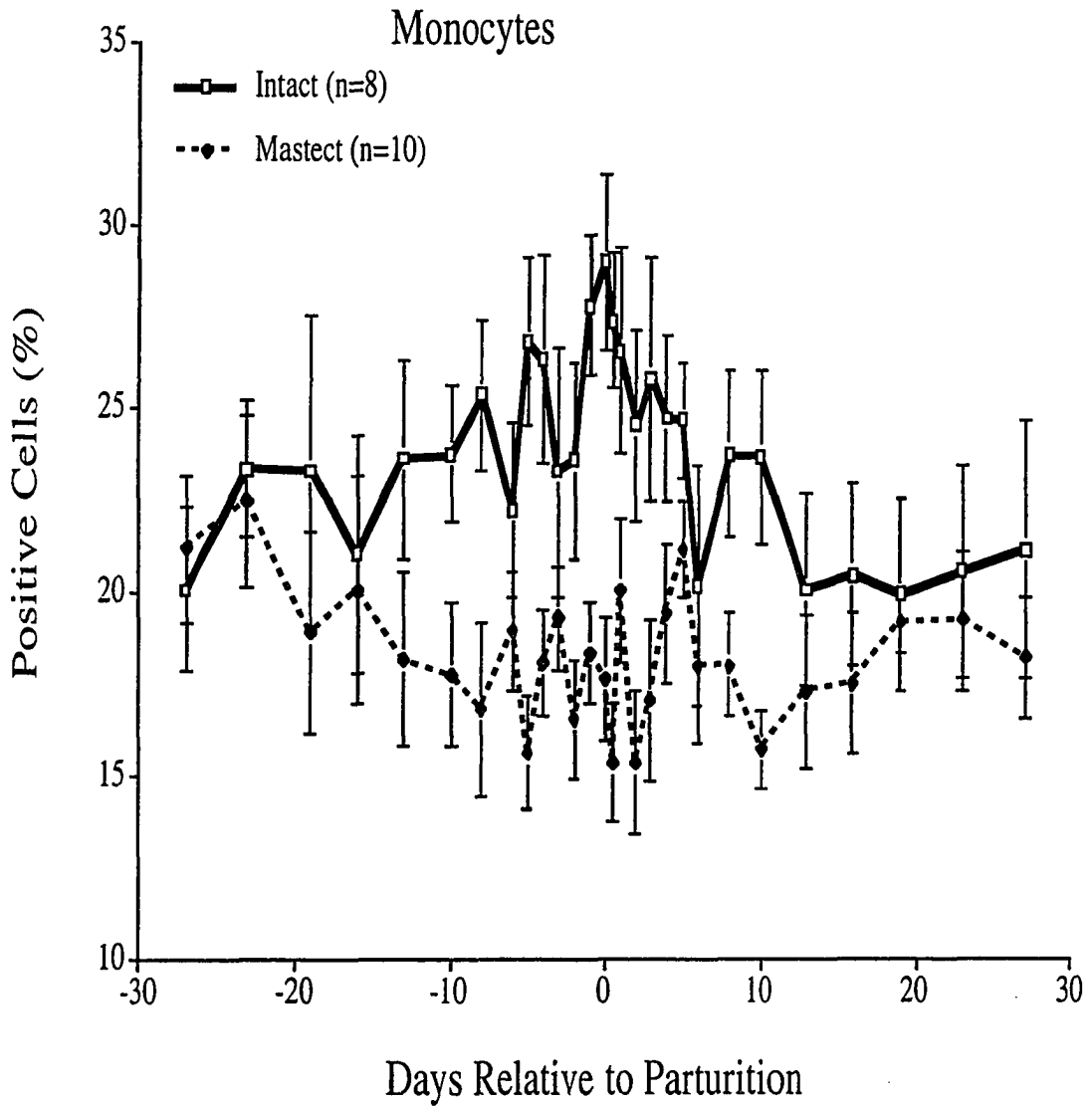Figure 10 -- Percentage of PBMC positive for the monocyte antigen in whole blood from intact and mastectomized cows during periparturient period.
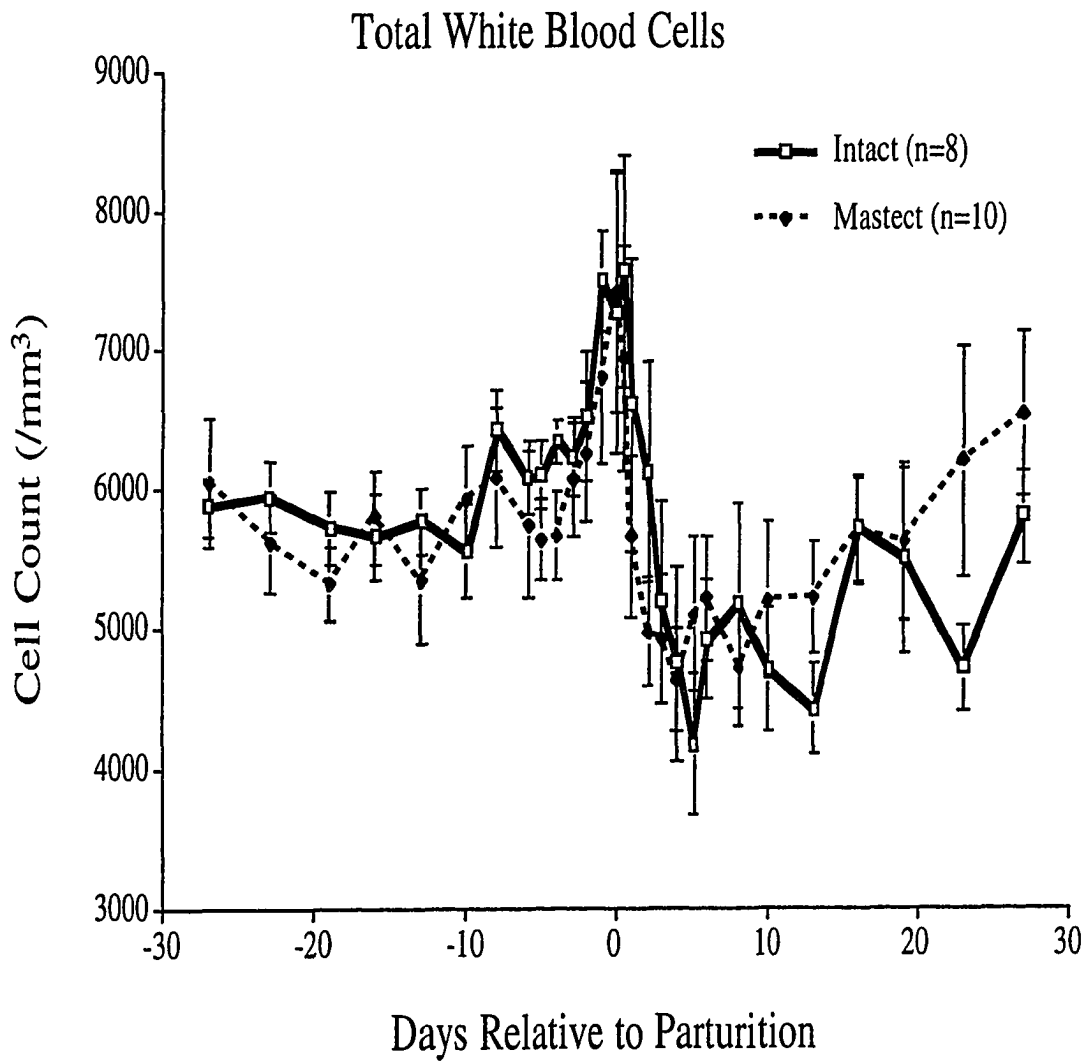
Total White Blood Cells

Figure 11 -- Cell counts of total WBC in whole blood from intact and mastectomized cows during periparturient period.
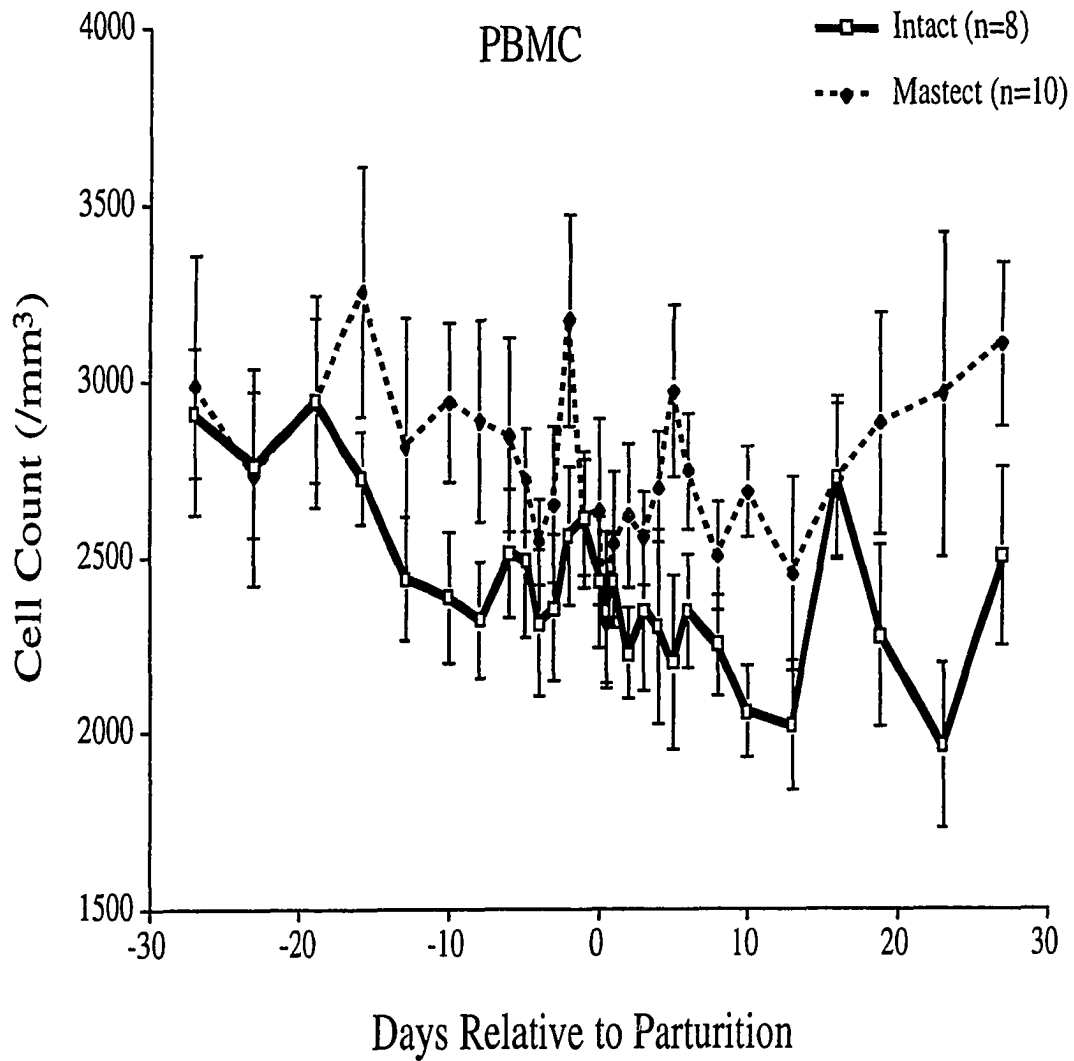
Figure 12 -- Cell counts of PBMC in whole blood from intact and mastectomized cows during periparturient period.

EFFECT OF THE PRESENCE OF THE MAMMARY GLAND ON EXPRESSION OF NEUTROPHIL ADHESION MOLECULES AND MYELOPEROXIDASE ACTIVITY IN PERIPARTURIENT DAIRY COWS

A paper submitted to the Journal of Dairy Science

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ABSTRACT

Neutrophil function is diminished in the periparturient period, especially in the dairy cow. Milk production negatively impacts energy, protein and calcium balance which may contribute to the severity of immunosuppression experienced by the cow around parturition. Using 10 mastectomized and 8 intact multiparous Jersey cows (all intact cows developed milk fever) we studied constitutive and platelet-activating factor activated expression of adhesion molecules ($\beta 2$ -integrins and L-selectin) on neutrophils by flow cytometry and assessed neutrophil myeroperoxidase activity during the periparturient period. Expression of $\beta 2$ -integrins in intact cows was highest at parturition. Expression of $\beta 2$ -integrins was greater in intact cows than in mastectomized cows throughout the study. L-selectin expression exhibited a sudden decrease at parturition with recovery within a day after parturition in both intact and mastectomized cows. The ability of neutrophils to kill microbes as assessed by neutrophil myeroperoxidase activity decreased before parturition in both groups. While there was a

quick recovery of neutrophil myeroperoxidase activity in mastectomized cows, there was no recovery in intact cows after parturition throughout the study which lasted until d 20 post partum. Milk production seems to exacerbate periparturient immunosuppression, especially with regard to recovery of neutrophil myeroperoxidase activity.

(**Key words:** immunosuppression, periparturient, neutrophils, the β2-integrin, L-selectin, myeroperoxidase)

Abbreviation Key: MPO = myeroperoxidase, **ACD** = acid citrate dextrose, **FACS** = flow cytometry, **PAF** = platelet-activating factor, **FITC** = fluorescein isothiocyanate, **MFI** = mean fluorescent intensity, **ICAM** = intercellular adhesion molecule, **WBC** = white blood cell, **ANOVA** = analysis of variance

INTRODUCTION

Impaired neutrophil and lymphocyte function during the periparturient period is a contributing factor to the high incidence of infectious disease observed in the periparturient cow (6, 10, 11, 12, 14, 15). The loss of immune function not only increases susceptibility to new infections leading to such diseases as mastitis and metritis (21, 22, 29), but also can allow subclinical infection by microbes causing diseases such as salmonellosis and paratuberculosis to become clinical.

Neutrophils are part of the innate immune system and therefore play an important role as the first line of defense against many infections. To be effective, the neutrophil must be capable of receiving chemical signals that the body has been invaded by a microbe and then must be able to egress from the blood stream at the site of microbial invasion. Neutrophils normally patrol endothelial surfaces, loosely binding to and rolling across the endothelium, ready to

leave the vascular system wherever bacteria have invaded the body. This rolling adhesion along endothelial surfaces is facilitated by the tethering interaction of L-selectin, a protein on the surface of neutrophils, with carbohydrate ligands on the vascular endothelium (17, 24). In the event of bacterial invasion of tissue, the endothelium of the post-capillary venules in that region will upregulate expression of several adhesion molecules including intercellular adhesion molecule (**ICAM**)-1 in response to inflammatory stimuli. ICAM-1 in addition to the constitutively expressed ICAM-2 on the endothelial surface will tightly bind proteins on the surface of neutrophils known as β2-integrins. This process allows neutrophils to become stationary long enough to migrate through the vascular endothelium. The expression of β2-integrins on neutrophils can be upregulated in response to chemotactic factors, such as complement fragment C5a, tumor necrosis factor, granulocyte-macrophage colony-stimulating factor, interleukin 8, and platelet activating factor (**PAF**). These same stimuli tend to down-regulate expression of L-selectin on neutrophil surfaces (17, 24, 28).

Monoclonal antibodies which specifically bind L-selectin and the β2 subunit of integrin allow quantitation of these proteins on neutrophils by flow cytometry (**FACS**). We have previously demonstrated a dramatic decrease in the expression of L-selectin at parturition in Holstein cows (19). Moreover, expression of β2-integrins on neutrophils increased gradually toward parturition, peaked at calving, then returned to initial values by 15 hours post partum both in resting and PAF-stimulated neutrophils (19).

Once neutrophils have left the vascular system they must migrate toward invading bacteria (chemotaxis), adhere to the bacteria by opsonization, ingest the bacteria, and finally kill the bacteria. In vitro tests of all of these functions of neutrophils have been developed and, when applied to neutrophils of periparturient cows, most studies suggest marked impairment of chemotaxis and ability of neutrophils to generate bactericidal agents (6, 11, 14). In our laboratory iodination of organic compounds catalyzed by neutrophil myeroperoxidase (**MPO**) is used as an index of the ability of neutrophils to kill ingested bacteria. In previous studies,

neutrophil MPO activity was greatly diminished during the periparturient period (6, 11, 14). Greater suppression of MPO activity was associated with infectious disease (6, 16).

The onset of milk production imposes tremendous challenges to the mechanisms responsible for energy, protein, and mineral homeostasis in the cow. Negative energy, protein, and/or mineral balance associated with the onset of lactation may be partially responsible for the immunosuppression observed in periparturient dairy cattle. Mastectomy of pregnant dairy cows removes the impact of milk production while presumably maintaining endocrine and other changes associated with late pregnancy and parturition. Mastectomy would be expected to improve immune function in the periparturient dairy cow, if milk production is an immunosuppressive factor. In this longitudinal study, we compared adhesion molecule expression on resting and PAF-activated neutrophils, and neutrophil MPO activity from 10 mastectomized and 8 intact periparturient Jersey cows in order to see the effect of lactation.

MATERIALS AND METHODS

Animals

Eighteen multiparous Jersey cows between 5 and 8 years of age were used in this study. Ten cows were mastectomized during mid gestation and allowed to heal. The cows calved between February 1996 and January 1997. Eight intact cows due to calve around the same time as each mastectomized cow were chosen as controls so that reagents used on mastectomized cow neutrophils were also used to assess neutrophil function of intact cows thus reducing the effects time and season might have on the outcome of the experiment. Cows were managed as similarly as possible and cared for by the same caretakers for the duration of the study. Cattle were housed in a free-stall barn until 2 to 3 d before calving, when they were

brought into maternity pens. They were fed alfalfa-corn silage based diets prior to and after calving with a dietary high cation-anion difference $[(Na^+ + K^+) - (Cl^- + S^{2-}) = + 400$ meq/kg]. This was done to increase the probability of milk fever in intact cows.

Blood samples were collected by jugular venipuncture into tubes containing acid citrate dextrose (**ACD**) for neutrophil assays. Samples were taken four wks before the expected calving date through four weeks after calving for adhesion molecule analysis, and for three weeks prior to three wks after calving for the neutrophil MPO assay. Bleeding frequency for adhesion molecule analysis and leukocyte count was twice a week for wks -4, -3, 3, and 4 around parturition; three times a week during wks -2 and 2; and daily during wks -1 and 1 around parturition. On the day of calving, samples were taken at 0, 12 and 24 hours after calving. For MPO activity assay, blood samples were taken from six cows from each group, twice a week. At each sampling time, blood samples from 4 non-pregnant heifers were taken to be used as internal laboratory controls for the MPO assay.

Enumeration of Leukocytes and Leukograms

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

L-selectin and β2-integrin expression on neutrophils

Monoclonal antibodies

Monoclonal antibodies against human L-selectin (Clone DREG-56, IgG1 Isotype, Pharmingen, San Diego, CA) and against the human β2 subunit of integrin (MHM23, LFA-1 β chain, monoclonal antibody, DAKO Corp, Caprinteria, CA) were used. An isotype control antibody (Clone X-927, IgG1 Isotype, DAKO Corp, Carpinteria, CA) was also used to correct for non-specific binding of antibody to the cells of interest. All antibodies were directly conjugated with fluorescein isothiocyanate (**FITC**) (2).

Activation of neutrophils and immunostaining

Quantitation of adhesion molecules on the surface of neutrophils was done in the resting state and following stimulation with PAF. A whole blood assay was used for the immunostaining of

L-selectin and β2-integrin on neutrophils. For each blood sample, 5 wells in a microtiter plate (U-bottom, 96-well) were dispensed with 50 μl aliquots. Ten microliters of PAF (10 μg/ml) were added to two wells to activate neutrophils and incubated for 5 min. at 39°C. Three wells were treated with 10 μl of PBS only and remained in the "resting" state. Two pairs of an unstimulated and a PAF-stimulated wells were treated with either 20 μl of anti-L-selectin antibody or 10 μl of anti-β2 subunit of integrin antibody. The remaining one unstimulated well was treated with 10 μl of isotype control antibody, and the plate was incubated for 15 min. at room temperature. Erythrocytes were lysed with 150 μl hypotonic lysing solution (2) for 90 s at room temperature. Then 75 μl of hypertonic restoring solution (2) was added to restore the solution to isotonicity. The supernatant was decanted after centrifugation at 800 x g for 2 min. This lysing process was repeated once more. The remaining leukocytes were

washed once with 200 μl of PBS, and the cells were suspended in 200 μl of sheath fluid
(Isoton II, Coulter Diagnostics, Hialeah, FL) for immediate FACS analysis.

Flow Cytometric Analysis

A flow cytometer (FAC Scan™, Becton Dickinson, San Jose, CA) was used to acquire and
analyze the neutrophil L-selectin and β2-integrin data. Data from 10,000 events per sample
were acquired and analyzed using a software (Cell Quest™ , Becton Dickinson, San Jose,
CA). The neutrophil population was gated out from the other leukocyte populations based on
their forward and side scatter characteristics on dot plots (2). The quantity of adhesion
molecules on each cell was expressed as the geometric mean of fluorescent intensity (MFI) in
the FITC fluorescence histogram.

Myeroperoxidase assay

The ability of cells to incorporate radioactive iodine (^{125}I) into trichloroacetic acid-
precipitable material was determined as previously described (26). The assay was performed
in cells stimulated with opsonized zymosan and in the absence of cells (to determine the extent
of nonspecific adsorption). Results were converted to nanomoles of ^{125}I incorporated/10^7
cells/hr and were converted to a percentage of internal laboratory control (heifers) for each
sampling day.

Statistical Analysis

For statistical purposes, data on adhesion molecule expression were grouped into weekly
periods (wks -4 to 4) except around the time of calving (d -1, 0, 0.5, and 1). The weekly

mean of individual animals was calculated from 2 (for wks -4, -3, 3, and 4), 3 (for wks -2 and 2) and 5 sampling times (for wks -1 and 1). For iodination, the data were arranged for d -20, -16, -12, -8, -4, 0, 4, 8, 12, 16 and 20. Each time the data were calculated from 1 to 3 sampling times. To investigate the impact of mastectomy on relevant variables we performed split-plot analyses of variance (ANOVA), followed by simple effect analyses in cases of significant (P<0.05) interaction. The non-repeated measure factor was treatment (intact vs. mastectomy) and the replication factor, cow. The repeated measure factor was time (days relative to parturition). (Because of some missing data, the ANOVA data were analyzed using the general linear model partial sums of squares procedure.)

RESULTS

General observations

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

Enumeration of leukocytes and leukogram

The total white blood cell (WBC) count increased prior to calving and decreased just after calving (P=0.0001). This change was primarily attributable to an increase in neutrophils

(P=0.0001). Intact and mastectomized cows showed similar changes in WBC count throughout the periparturient period of study (Figs. 1 and 2).

Flow cytometric analysis

Basal and PAF-stimulated neutrophil expression of L-selectin are presented in Fig 3 and Fig 4. Both mastectomized and intact cows exhibited a similar sudden decrease in expression of L-selectin at parturition followed by an increase after parturition. ANOVA showed time (P=0.02) and treatment x time interaction (P=0.001) effects but no treatment (P=0.977) effect. PAF activation had no significant effect on L-selectin expression when compared to resting L-selectin expression.

In contrast to L-selectin, β2-integrin expression showed a completely different pattern (Figs 5,6). In intact cows, β2-integrin expression was up-regulated at parturition (from d -1 to d 1). This was followed by a down-regulation after parturition to a level below prepartum values, and remained below prepartum levels throughout the postpartum sampling period. Mastectomized cows showed slightly lower expression of β2-integrin after parturition without significant up-regulation at parturition and tended to be lower than intact cows throughout the sampling period. PAF stimulation in vitro resulted in 40 - 60 % upregulation of β2-integrin expression. However, β2-integrin expression following PAF stimulation exhibited the same pattern as constitutive expression and there was no evidence of a difference in responses to PAF between intact and mastectomized cows. ANOVA showed significant treatment (P=0.04), time (P=0.0001), and treatment x time interaction (P=0.009) effects on β2-integrin expression.

Myeroperoxidase assay

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

DISCUSSION

Neutrophils play an important role in protection from infectious diseases such as mastitis (9, 27), but this function is greatly diminished in periparturient dairy cows (6, 11, 30). In this study we found that milk production affects β2-integrin expression and recovery of MPO activity in neutrophils after calving but does not seem to be a factor in leukocyte cell count and L-selectin expression on neutrophils.

The leukocyte cell count showed similar changes in both groups which were attributable mostly to transient changes in neutrophils which agrees with previous reports (8, 12, 13). As both groups showed similar increases in neutrophil count and decreases in L-selectin expression at parturition, the increase in neutrophils at parturition may be due to the decreased neutrophil margination associated with down-regulated L-selectin expression, thus increasing the circulating pool of neutrophils. This acute transient loss of neutrophil rolling would likely reduce egress into tissue in response to infection. Transient loss of neutrophils available for egress into diseased tissues can have severe consequences (9, 27).

The pattern of L-selectin expression on neutrophils was quite similar to our former study in intact cows (19) and there was no lactation effect. It suggests that the stress of parturition

decreases L-selectin expression which could potentially reduce the initial tethering of neutrophils necessary for rolling on endothelial cells and thus impede migration of neutrophils to sites of infection when needed.

$\beta2$-integrin expression showed a clear effect of lactation in periparturient cows. The results from intact cows were similar to our previous study (19), however, $\beta2$-integrin expression after parturition did not recover and remained lower than the pre-parturient value in the present study. This may reflect the influence of metabolic diseases which developed after parturition in all intact cows. Interestingly mastectomized cows showed lower expression of $\beta2$-integrin throughout the sampling period. Thus, the dramatic change and higher expression during the sampling period in intact cows depends on the presence of the mammary gland and lactation. It is difficult to explain the significance of $\beta2$-integrin expression seen in intact cows. Because neither the constitutive nor the induced integrins are fully functional (4, 18, 23, 33). Higher expression does not necessarily mean higher adhesiveness by integrins. Stimulation by chemoattractants and divalent cations are necessary for integrins to be functional (1, 5).

The emigration of neutrophils from the vasculature is regulated by at least three distinct steps. The first step is a "capture" and "rolling" adhesion mediated by the selectins. After this initial step, the leukocyte must be activated by chemoattractants, and the third step is firm adhesion or "arrest" of the cells to endothelium by the activated integrins (20). A key feature is that these steps act in sequence, not in parallel. The inhibition of any one of these steps gives essentially complete, rather than partial, inhibition of neutrophil emigration (32).

Colostrum contains many leukocytes including neutrophils. The up-regulation of $\beta2$-integrins on neutrophils before parturition may be related to the migration of neutrophils into mammary glands in intact cows. Since L-selectin was down-regulated at parturition, neutrophil migration may have been transiently diminished at parturition. In contrast to $\beta2$-integrins, L-selectin is in a constitutively active state (3), although its affinity for ligands can

be increased after stimuli (31). Down-regulation of L-selectin inhibits egress of neutrophils even if there is a significant up-regulation of β2-integrins.

The iodination reaction is dependent upon a complex chain of events; including, ingestion, oxidative metabolism, degranulation, and MPO activity. Thus, the in vitro iodination reaction is a good screening test to evaluate neutrophil function (26). In previous studies (6, 7, 11, 14), the MPO activity of bovine neutrophils begins to decline around 2 wk prepartum, reaches a nadir during the first 7 to 10 d postpartum and then takes about 4 wk to recover to 3 wk prepartum levels. This is similar to our present study of intact cows. Impairment of neutrophil iodination is critical because poor neutrophil MPO activity has been associated with an increased incidence of infectious diseases of the mammary gland and reproductive tract in postpartum dairy cows (6, 7, 16), as well as respiratory disease (25). Although both groups in the current study exhibited a decline in MPO activity before parturition, mastectomized cows recovered quickly whereas intact cows did not recover even at d 20 postpartum. This suggests that factors leading to the processes associated with parturition impair neutrophil MPO activity, and milk production markedly delays recovery of this function. The poor recovery of neutrophil function may due to the negative energy, protein, and/or mineral balances associated with lactation.

CONCLUSIONS

The effect of milk production on adhesion molecule expression and function of neutrophils was studied using mastectomized cows in comparison with intact cows. L-selectin expression did not seem to be affected by milk production exhibiting a sudden decrease at parturition with recovery within a day after parturition in both intact and mastectomized cows. Expression of β2-integrins was influenced by milk production with higher expression in intact cows throughout the sampling period especially around parturition. The MPO activity of neutrophil

declined before parturition in both groups. While MPO activity quickly recovered in mastectomized cows after parturition, there was no recovery in intact cows throughout the study. For a neutrophil to be effective as the first line of defense against infection, it is necessary to have a harmonious process of migration, ingestion and killing. Lack of any of these processes results in a diminished function of neutrophils. Therefore, even if higher expression of β2-integrins occurs in intact cows, prolonged reduction in neutrophil MPO activity seen after calving in intact cows may diminish host defense. From these results, we conclude that milk production exacerbates periparturient immunosuppression, especially with regard to recovery of neutrophil function after calving. The specific factors causing this effect are not known but could include various hormones affecting lactation or the negative energy, protein and mineral balances of postpartum dairy cows.

ACKNOWLEDGMENTS

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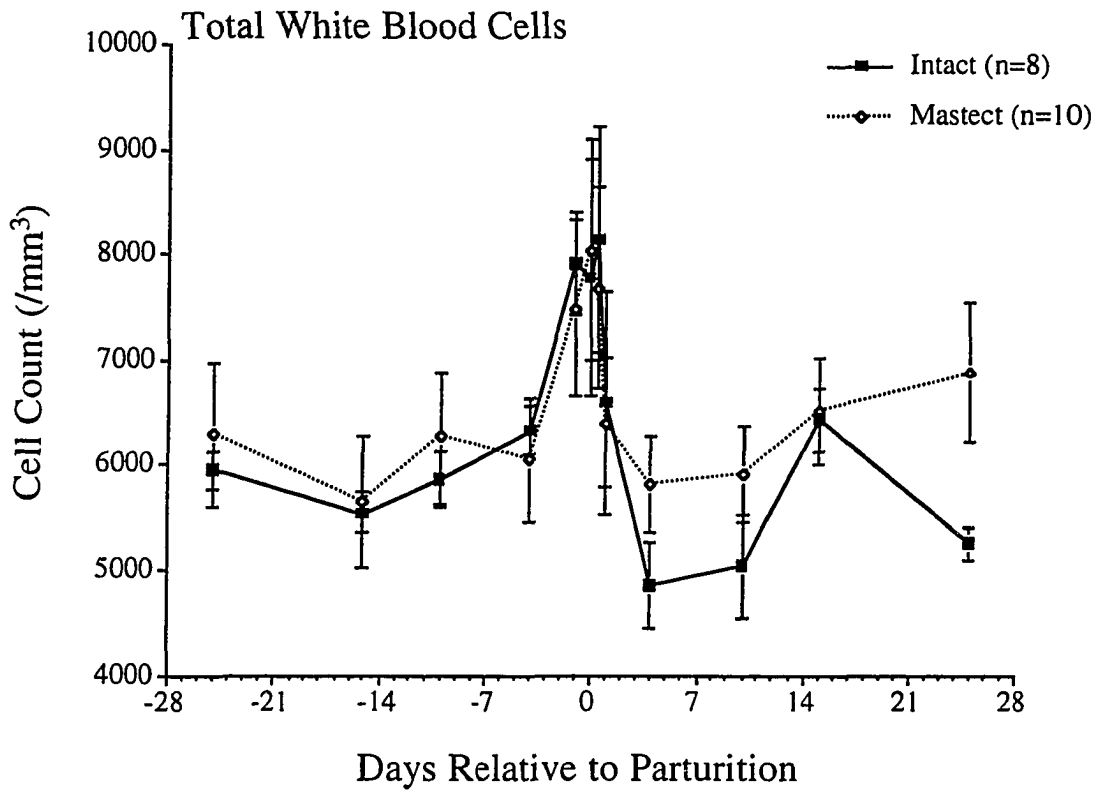
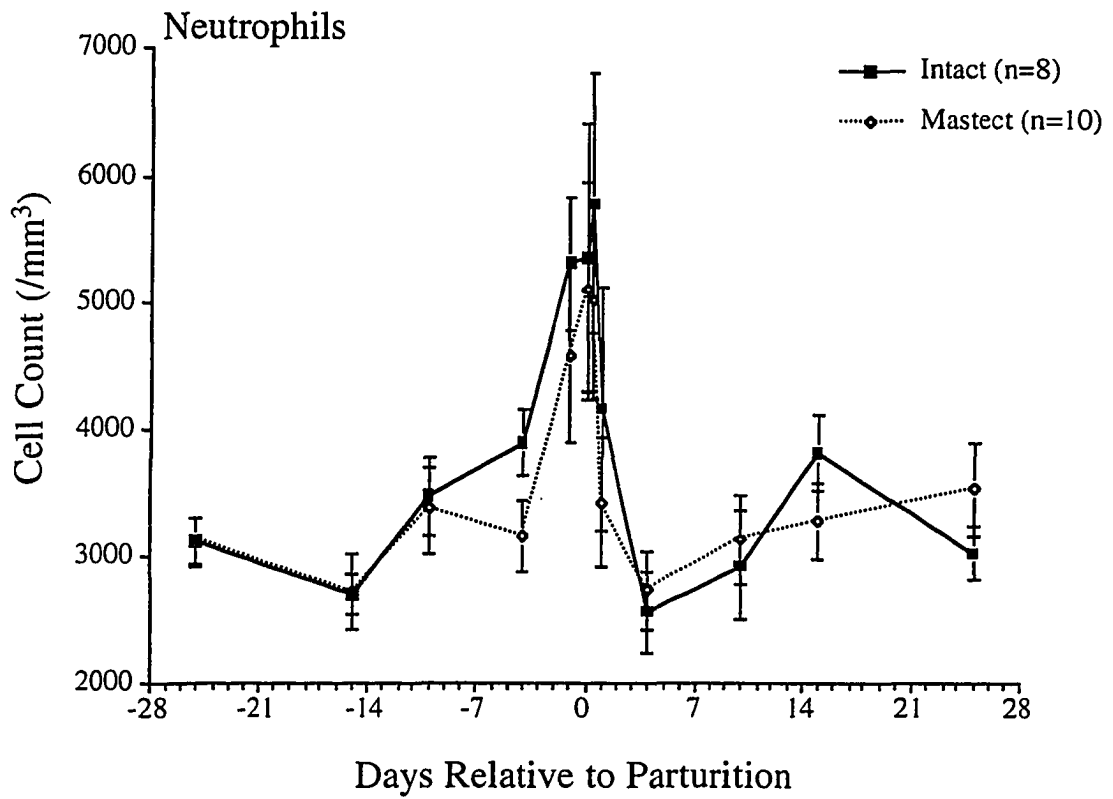Figure 1. Cell count of total leukocytes in whole blood (/mm³) of periparturient Jersey cows (mean ± SEM).

Figure 2. Cell count of neutrophils in whole blood (/mm^3) of periparturient Jersey cows (mean ± SEM).
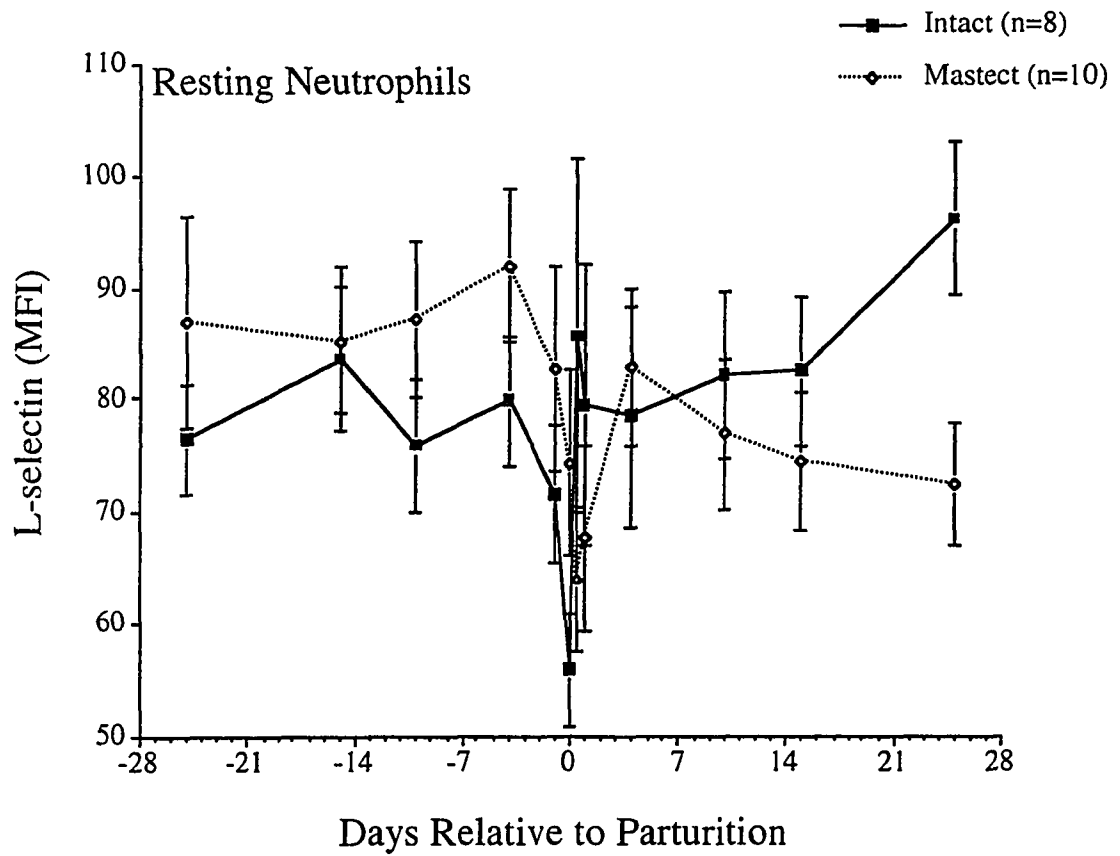
Figure 3. Basal expression as measured by mean fluorescent intensity (MFI) of L-selectin
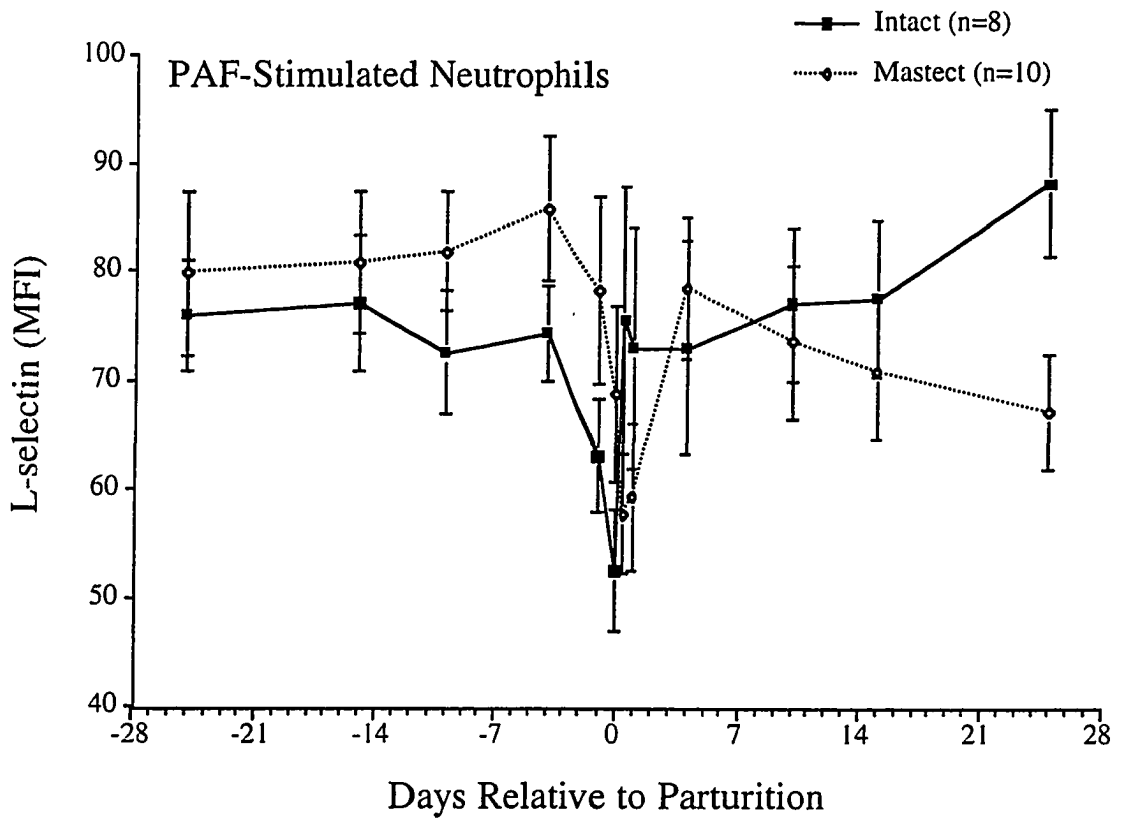
on neutrophils in periparturient Jersey cows (mean ± SEM).

Figure 4. PAF stimulated expression as measured by MFI of L-selectin on neutrophils in periparturient Jersey cows (mean ± SEM).

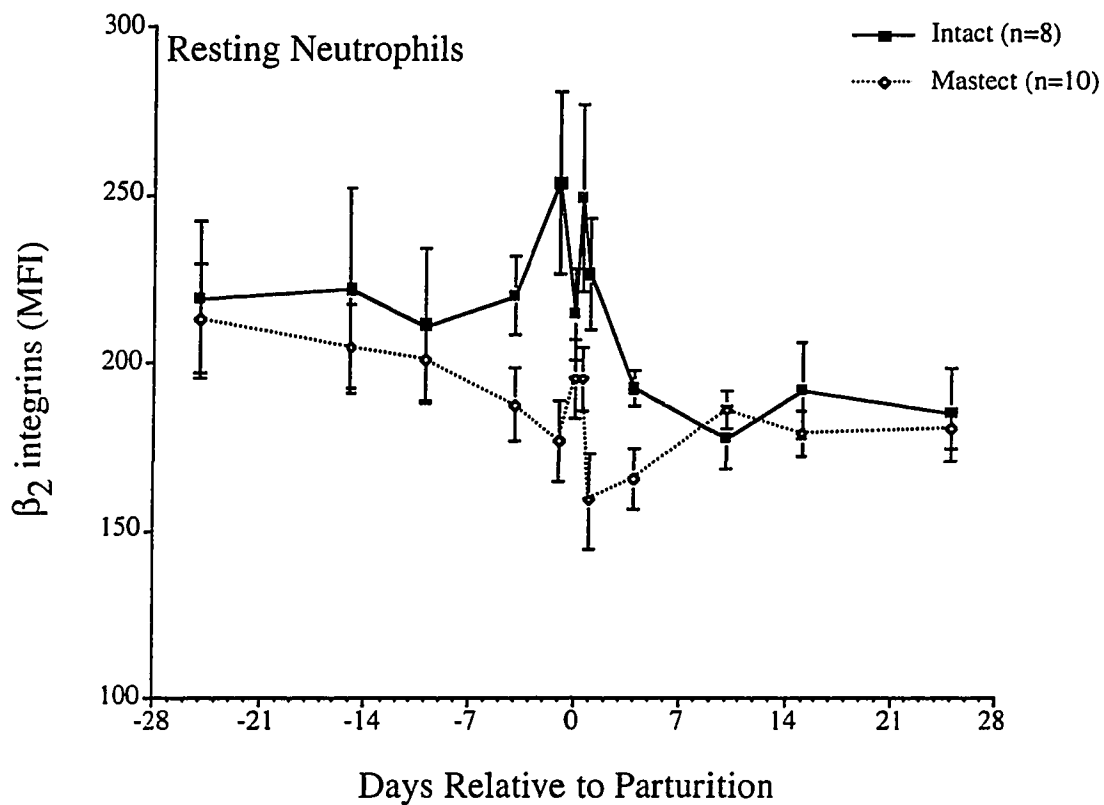Figure 5. Basal expression as measured by MFI of β2-integrin on neutrophils in
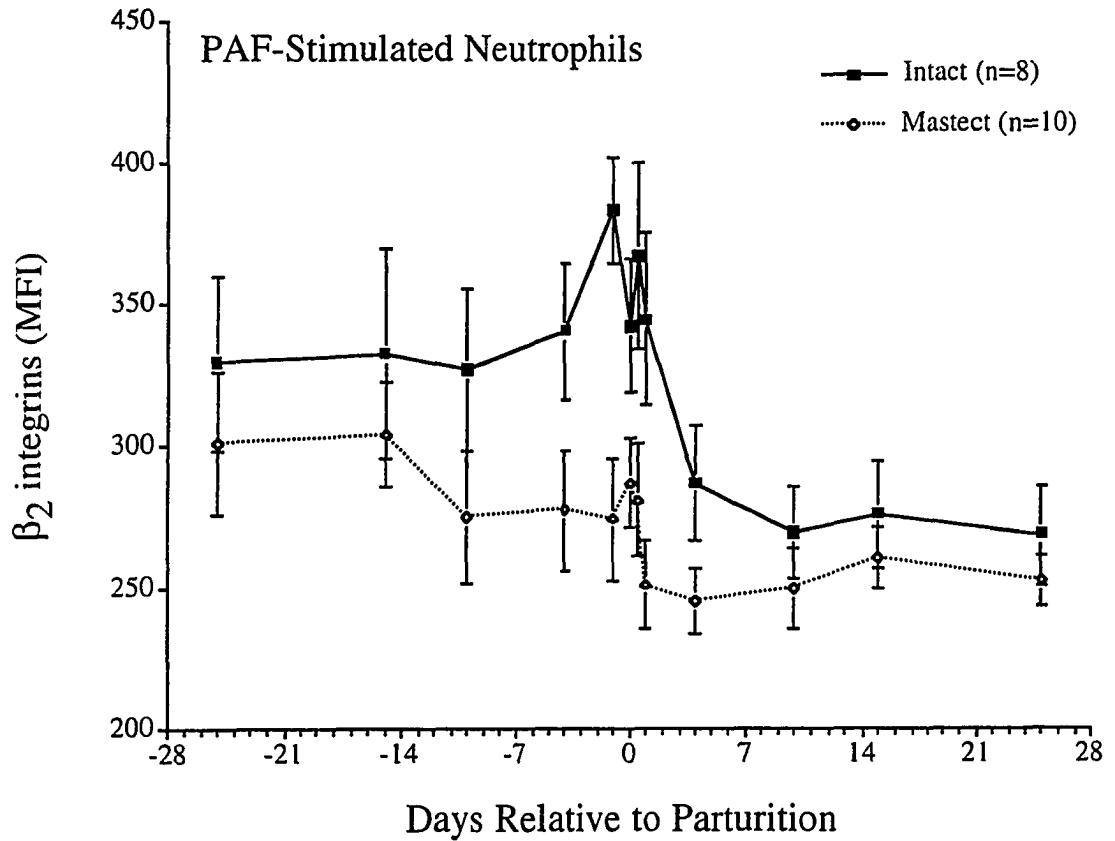
periparturient Jersey cows (mean ± SEM).

Figure 6. PAF stimulated expression as measured by MFI of β2-integrin on neutrophils in periparturient Jersey cows (mean ± SEM).
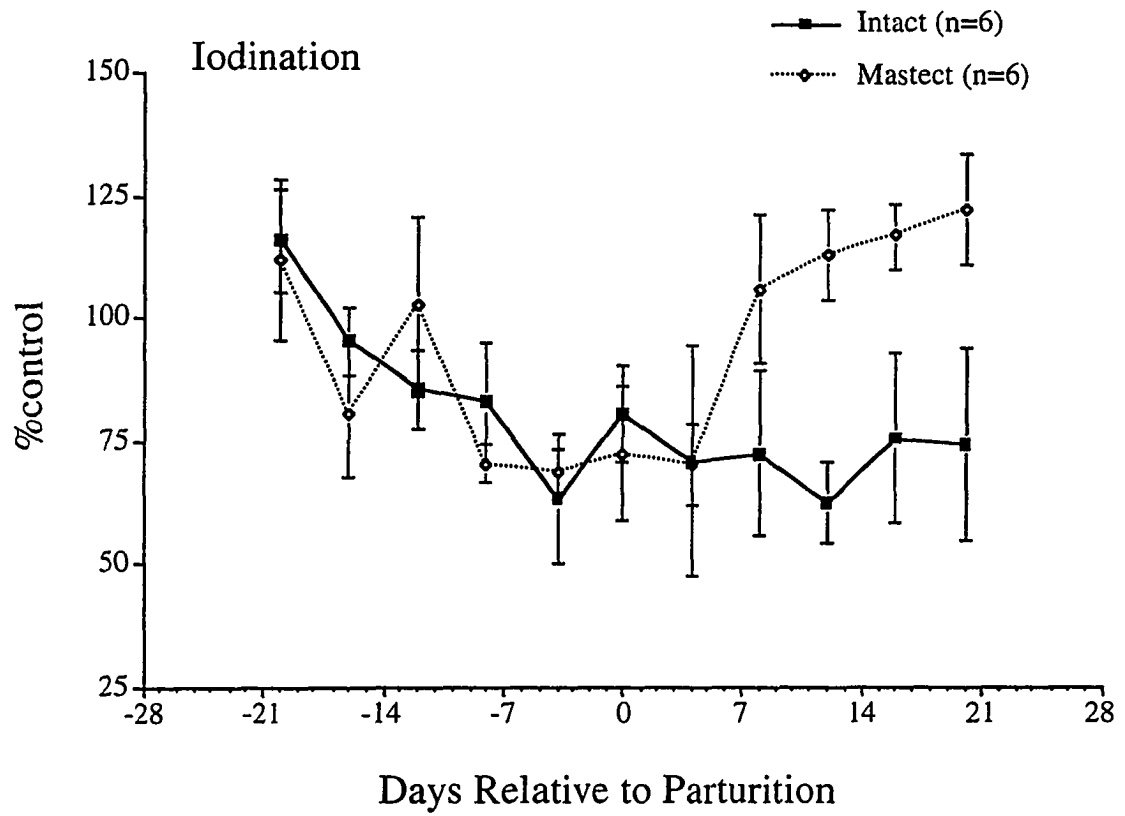
Figure 7. Neutrophil iodination capacity in periparturient Jersey cows expressed as a

percentage of laboratory control (mean ± SEM).

EFFECTS OF THE PRESENCE OF THE MAMMARY GLAND ON STEROID HORMONES IN PERIPARTURIENT DAIRY COWS

A paper to be submitted to the Journal of Dairy Science

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ABSTRACT

Periparturient immunosuppression in dairy cows is thought to be caused by high level of steroid hormones and/or negative energy balance associated with parturition and colostrum synthesis. Our mastectomized cow studies showed that lactation and the mammary gland play an important role in periparturient immunosuppression since mastectomy eliminates almost all immunosuppressive changes except the decline in neutrophil myeloperoxidase activity before parturition. In order to see the effect of colostrum production and mammogenesis on steroid hormone profiles in association with immune function, we used 6 intact and 6 mastectomized multiparous Jersey cows. Estrone and estradiol showed a marked linear increase from day - 10 to day 0 of parturition and dropped rapidly to low levels immediately postpartum in both intact and mastectomized cows. Estrone was significantly higher in mastectomized cows compared to intact cows during the sampling period whereas estradiol did not show the treatment (mastectomy) effect in the overall sampling period although it tended to be higher

from d -10 to -5. Progesterone showed the same pattern and level in both groups. The cortisol level was higher at the beginning of sampling (from d -14 to d -7) in mastectomized cows but both groups showed similar changes after d -4 and there was no treatment effect. These data suggest that a high level of steroid hormones does not seem to be responsible for the further impairment of immune function of PBMC seen in periparturient dairy cows although high levels of estrogens and cortisol may have a certain effect on iodination capability and L-selectin expression in neutrophils. The reason for this periparturient immunosuppression remains unclear.

(**Key words:** immunosuppression, periparturient dairy cows, estradiol, estrone, progesterone, cortisol)

Abbreviation Key: PBMC = peripheral blood mononuclear cells,

INTRODUCTION

Periparturient immunosuppression in dairy cows is manifested in the high incidence of disease immediately after parturition. It has been demonstrated that this high incidence of disease is due to the impaired immune function both in peripheral blood mononuclear cells (**PBMC**) (10, 12) and neutrophils (4, 11), which are associated with a decline in the T cell population in cows during this period (13, 27). However, the reason for this immunosuppression is still unknown. During this period, cows are exposed to tremendous physiological changes associated with parturition and colostrum production, and both factors can cause immunosuppression. We wondered how milk production contributes to this periparturient immunosuppression. In order to see the effect of colostrum production, we used mastectomized cows in comparison with intact cows and analyzed immune cell

population and function. We found that mammogenesis and colostrum production play an important role in this immunosuppression. Mastectomy eliminated the most of the changes in immune cell population during the periparturient period, i.e., a decline in T cell subsets and an increase in monocytes associated with the decline in lymphocyte function (in vitro production in interferon-gamma and IgM). Mastectomy did not change the decline in myeloperoxidase activity of neutrophils before parturition but allowed quick recovery to the preparturient value after parturition compared to no recovery in intact cows. Although we could demonstrate the importance of colostrum production in periparturient immunosuppression, the exact reason for this immunosuppression is still unknown.

Many researchers speculate that negative energy balance and a dramatic change in hormones associated with the parturition and lactation process may be contributing factors. Estrogen levels increases markedly at the end of gestation starting from last 3 wk of gestation and reaches its zenith at parturition (26, 29). The progesterone level remains high during pregnancy, then it drops precipitously just prior to calving (29). The cortisol level surges around parturition (d -2 to 2) but remains nearly constant during the last trimester of gestation (29). High levels of these steroid hormones are known to have immunosuppressive effects (7, 18, 23, 24, 25, 30), thus they are thought to be responsible for periparturient immunosuppression. Since mastectomy eliminated most changes in the immune cell population, and function, we wondered if there was a difference in steroid hormone levels between intact and mastectomized cows. To answer this question, we used 6 intact and 6 mastectomized cows to trace the effect of mastectomy on estradiol, estrone, progesterone, and cortisol; hence the relationship between higher level of steroid hormones during parturition and periparturient immunosuppression.

MATERIALS AND METHODS

Animals

Twelve multiparous Jersey cows between 5 and 8 years of age were used in this study. Six cows were mastectomized during mid-gestation and allowed to heal. The cows calved between February and July 1996. Six intact cows, due to calve around the same time as each mastectomized cow were chosen as controls. The cows were managed as similarly as possible and cared for by the same caretakers for the duration of the study. Cattle were housed in a free-stall barn until 2 to 3 d before calving, when they were brought into maternity pens. They were fed alfalfa-corn silage based diets prior to and after calving with a high cation-anion difference.

Plasma samples were obtained once a day (between 13:00 to 13:30) from 2 wk before calving till 2 wk after calving. Blood was collected from the jugular vein into a heparinized syringe. Plasma was separated from blood cells by centrifugation and placed in a -20°C freezer within 1 h of collection.

Hormone Assays

All hormones were assayed by radio immunoassay using commercial kits (Estrone DSL-8700, Estradiol DSL-4400, Progesterone DSL-3900: These are all from Diagnostic System Laboratories, Webster, TX. Coat-A-Count Cortisol from Diagnostic Product Corporation, Los Angels, CA) following to the company's instruction. Referring to the previously reported data, samples were chosen to reflect the changes in each periparturient period. That is, samples from d -10, -7, -4, -3, -2, -1, 0, 1, and 2 were used for an assay of estrone, samples from d -10, -7, -5, -3, -2, -1, 0, 1, and 2 were used for estradiol, samples from d -7, -5, -4, -

3, -2, -1, 0, 1, and 2 were analyzed for progesterone, and samples from d -10, -4, -3, -2, -1, 0, 1, 2, and 3 were used for cortisol analysis. Samples from two cows from each group were processed together to minimize the assay variation between groups and among samples. Radioactivity was counted on a gamma counter (Auto-Gamma 5780; Packard Instrument Co., Downers Grove, IL). Test sample counts were compared against those of standards supplied with the kits and the concentration (pg or ng/ml) of each steroid was determined from a single standard curve. The intra-assay coefficient of variations for the estrone, estradiol, progesterone, and cortisol were 3.63, 1.67, 3.11 and 4.31 % respectively. Before these analyses we did the validation of assays using serially diluted samples which are supposed to have a higher value in each steroid and they showed parallel relation with the standard curve for each steroid.

Statistical Analysis

In order to investigate the impact of mastectomy on relevant variables we performed split-plot analyses of variance , followed by a simple effect analyses in cases of significant ($P<0.05$) interaction. The non-repeated measure factor was treatment (intact vs. mastectomy) and the replication factor, cow. The repeated measure factor was time (days relative to parturition).

RESULTS

General Observations

Two mastectomized cows gave birth to twins. All intact cows developed milk fever within 24 hours after calving and they were treated with transfusion of calcium solution one to three

times. Three intact cows also developed ketosis and displaced abomasum within one week after calving and these were treated by intravenous glucose infusion and abomasopexy using a single suture inserted through the right ventral abdomen while the cow was held in dorsal recumbency.

Plasma Level of Estrone

Both groups showed a marked linear increase toward parturition and a sudden decrease at parturition. Estrone reached a zenith on day -2 with 806 ± 63 (mean $\pm$ SEM) in intact and 1516 ± 57 pg/ml in mastectomized cows (Figure 1). There was a significant difference between the groups before parturition where mastectomized cows had nearly twice as much estrone in plasma. Statistical analysis showed all the treatment (p = 0.0001), time (p = 0.0001), and treatment*time (p = 0.0001) effects.

Plasma Level of Estradiol

Estradiol also showed typical change at around calving in both groups (Figure 2). It increased significantly before parturition, then dropped precipitously after parturition showing a zenith on d -1 in intact and d 0 in mastectomized cows with 169 ± 23 and 232 ± 45 pg/ml respectively. Although mastectomized cows showed a higher value at the beginning of sampling, overall there was no treatment effect (p = 0.134). There is a significant time (p = 0.0001) and time*treatment
(p = 0.098) effects.

Plasma Level of Progesterone

Progesterone levels remained constant during the sampling period in both groups (Figure 3).until d -2, varying only 6000 to 70000 pg/ml. It dropped suddenly on d -1 to 1 to 2 ng/ml, then it became 0.1 to 0.2 ng/ml after calving. Statistical analysis showed no treatment (p = 0.604) and treatment*time (p = 0.935) effects, but there was a significant time effect (p = 0.0001).

Plasma Level of Cortisol

Although the cortisol level tended to be higher in mastectomized cows at the beginning of sampling (from d -14 to d -7) (Figure 4), starting with d -4, both groups showed a similar level with a sudden increase at parturition and decrease after parturition. Zenith was seen on d 0 with 19.4 ± 4.4 in intact and 14.5 ± 4.6 ng/ml in mastectomized cows. Overall, the difference between both groups was not statistically significant (treatment effect : p = 0.502, time effect : p = 0.002, treatment*time effect : p = 0.113).

DISCUSSION

This study showed that mastectomy did not decrease the level of steroid hormones; there was an increase in the estrogen level. The implication is that an increased steroid level during the periparturient period seems to be associated with the parturition process, although higher levels of steroid hormones are required for the initiation of lactation (5, 8, 28). A higher level of estrogen in mastectomized cows is contradictory to the report on mastectomized goats (22). This review paper summarized the importance of the mammary gland in the endocrine system, and it showed that the increase in estradiol concentrations, which occurs near term in intact

goats, was completely obliterated in mastectomized goats. This paper also introduced examples of infertility or problems associated with parturition in mastectomized animals due to the deficiency of estrogens from mammary gland. Estrogen secretion from the mammary gland in Jersey cows was also reported (31). We did not experience dystocia in any cows in this study. Gestation period, and parturition process did not differ between intact and mastectomized cows. A higher level of estrogen in mastectomized cows, or rather a lower level of estrogen in intact cows may be due to the increased influx of estrogen accompanied to blood flow into the mammary gland (6, 19).

Cows are immunosuppressed during pregnancy like other mammals to maintain the "foreign tissue, the fetus" inside (17). This pregnancy-associated immunosuppression is thought to be due to the high level of estrogen and progesterone. Many reports describe the relationship between high levels of estrogen and progesterone and maternal immunosuppression (14, 15, 25, 32). Both of these hormones are known to have immunosuppressive effects both in neutrophils and PBMC (7, 20, 30, 33). Although progesterone level remains relatively constant during pregnancy, these levels drop precipitously just prior to calving (29). The estrogen (both esterone and estradiol) level shows a marked linear increase from about 1 m before calving and it drops suddenly on the day of calving (26, 29). Our data, with selected samples in intact cows, showed a pattern similar to the reported studies. Estrogen levels in intact cows of this study tended to be lower than reported data. This may be due to the use of commercial kits designed for human samples. Bovine plasma may contain substances which disturb the binding of antibodies to estrogens.

Studies of immune function in periparturient dairy cows showed a linear decline in both neutrophil and PBMC functions (9, 11, 12, 21). These changes seem to be inversely correlated to the linear increase in estrogens. Therefore, it has been speculated that a higher level of estrogens is responsible for these immunological impairments. The absence of greater immunosuppression in mastectomized cows with higher estrogen levels suggests that if

estrogen is the main inducer of immunosuppression then it has saturated its capacity in the intact cows and cannot further suppress. In our other studies, we showed that mastectomy eliminated almost all changes in PBMC population (decrease in T cell population and increase in monocytes at parturition) and the function (decline in vitro IgM and IFN-γ production at parturition). However, mastectomized cows showed similar changes in L-selectin expression (sudden decline at parturition) and decline in myeloperoxidase activity before parturition in neutrophils as seen in intact cows. Although myeloperoxidase activity quickly recovered to preparturient values in mastectomized cows, intact cows showed no recovery until d 20. Mastectomized cows exhibited higher or similar level of steroid hormones compared to immunosuppressed intact cows. Therefore, the higher level of steroid hormones is not associated with the change in PBMC population and a decline in function. Since neutrophil function showed a similar decline in both intact and mastectomized cows before parturition, we cannot deny the effect of steroid, especially estrogens on neutrophil myeloperoxidase activity, since the decline of neutrophil myeloperoxidase activity seemed to be negatively correlated to a linear increase in estrogens.

Cortisol is the most well known immunosuppressive steroid. There are many reports about the relationship between high cortisol levels and diminished immune cell function (1, 2, 3, 9, 23). Surprisingly, mastectomized cows showed a similar or higher level of cortisol compared to intact cows in this study. This suggests that an existent impaired immune cell function due to pregnancy especially in PBMC function is not further suppressed by the sudden increase in cortisol at the time of calving since mastectomized cows show no further decline in PBMC function at the time of calving. The sudden increase in cortisol may well be associated with the down regulation of L-selectin and hence the increase in neutrophil numbers (16). Our mastectomized cow study of adhesion molecules also showed similar changes in intact cows, and this sudden down regulation of L-selectin and the subsequent increase in neutrophils were also seen in mastectomized cows. Since the myeloperoxidase activity reached a nadir at

parturition in both groups, it may be due to the immunosuppressive effect of cortisol which substitute the declined estrogen level in addition to the residual effect of estrogens.

In addition to steroid hormones mentioned above, mammogenesis and colostrogenesis require an increase in many kinds of hormones and nutrition. All factors can have an effect on immune function. They may be responsible for the periparturient immunosuppression especially for a decline in PBMC function and change in immune cell population.

CONCLUSIONS

In this study, we confirmed the dramatic change in steroid hormones in cows during periparturient period is mainly associated with the parturition process rather than colostrum production, although steroid hormones are necessary to initiate mammogenesis. Higher levels of estrogens in mastectomized cows, or rather lower levels of estrogens in intact cows may be due to the influx of estrogens into the mammary gland prior to parturition. The increase in steroid hormones does not seem to contribute to the periparturient immunosuppression in PBMC, although the increased levels of estrogens and cortisol may have a certain effect on the neutrophil function and decreased L-selectin expression at parturition. Further study is needed to elucidate the hidden causes of periparturient immnosuppression in dairy cows.

ACKNOWLEDGMENTS

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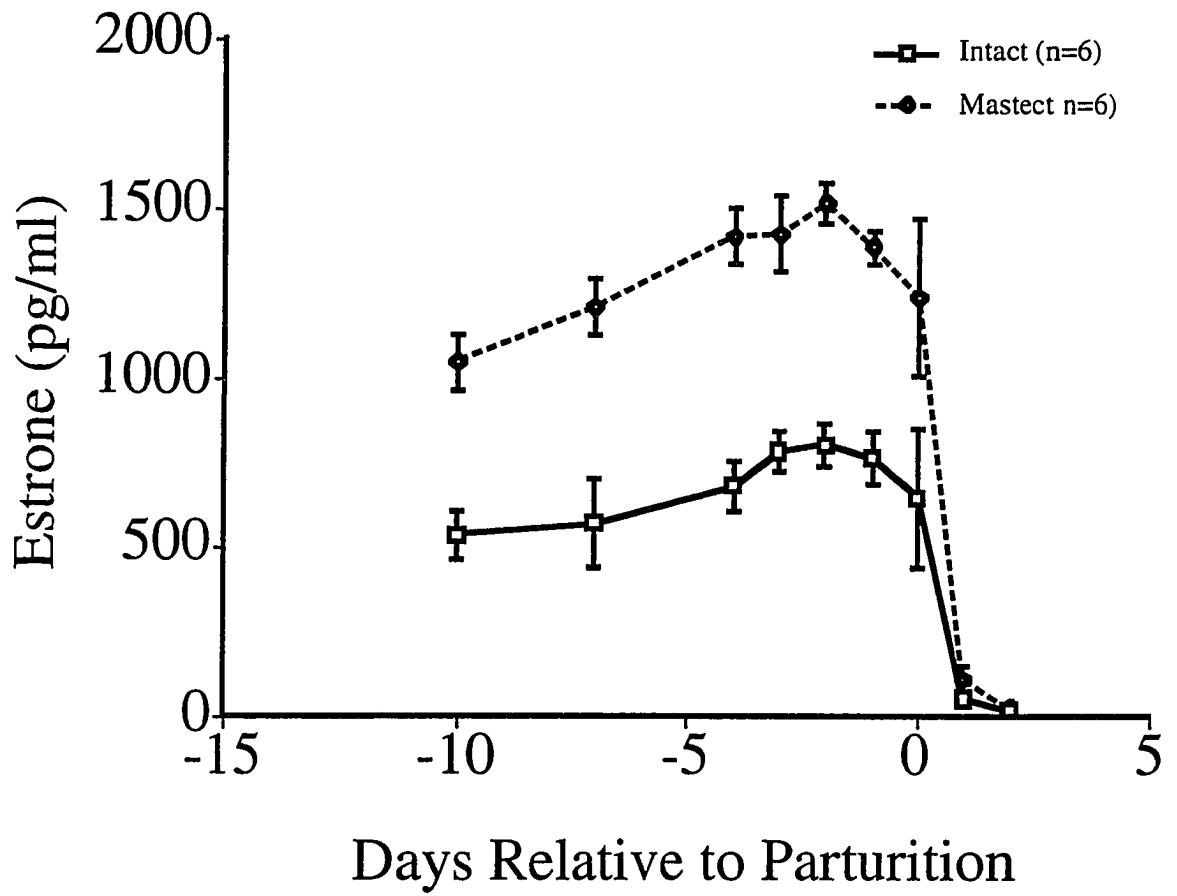
Figure 1. Plasma estrone level in intact and mastectomized cows during periparturient period.
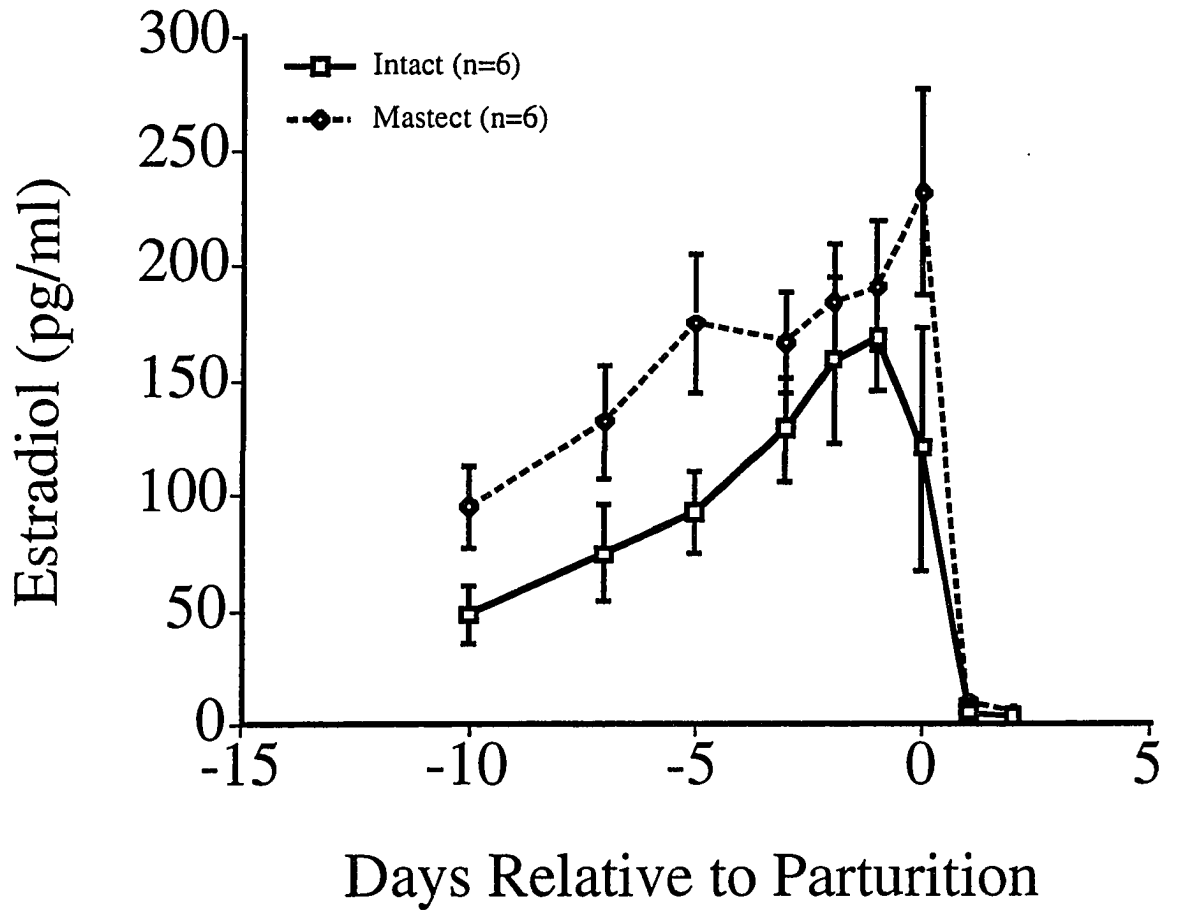
Figure 2. Plasma Estradiol level in intact and mastectomized cows during periparturient period.
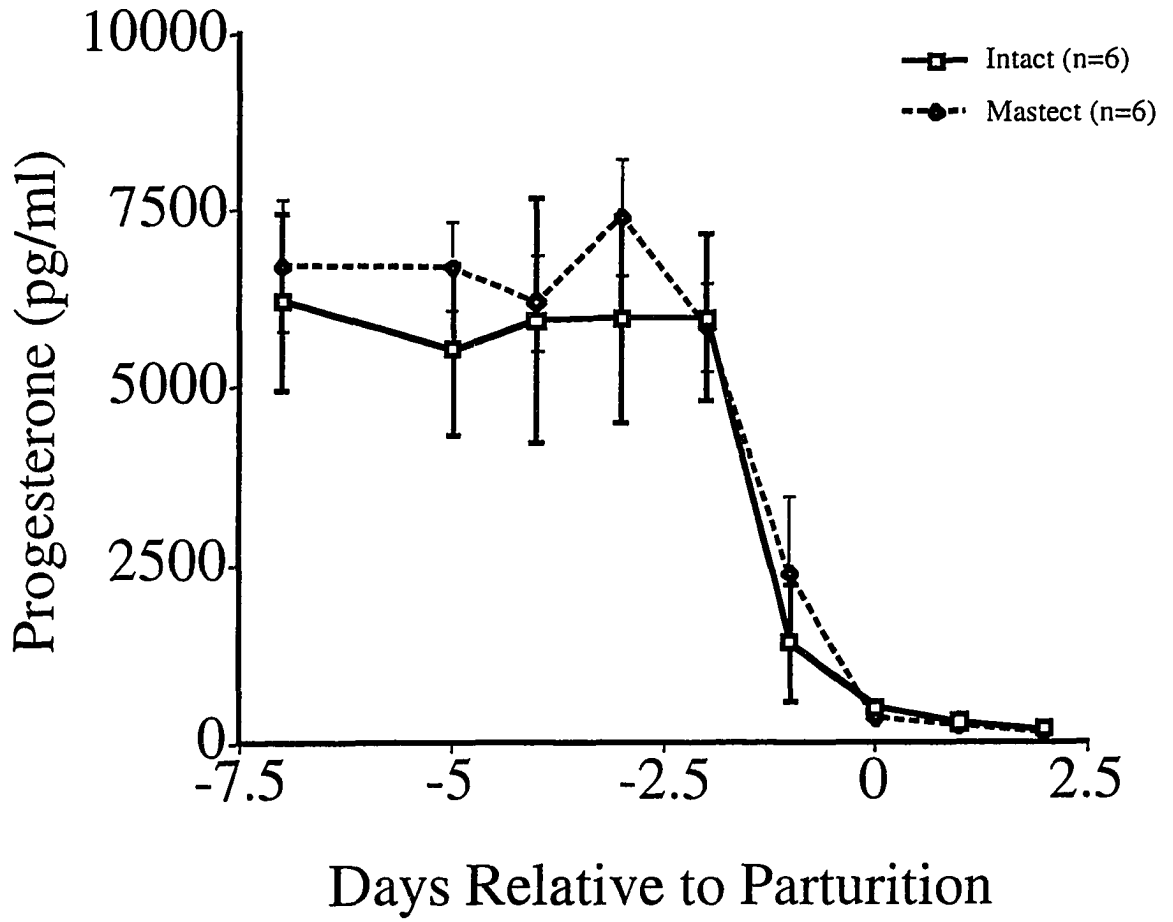
Figure 3. Plasma Progesterone level in intact and mastectomized cows during periparturient period.
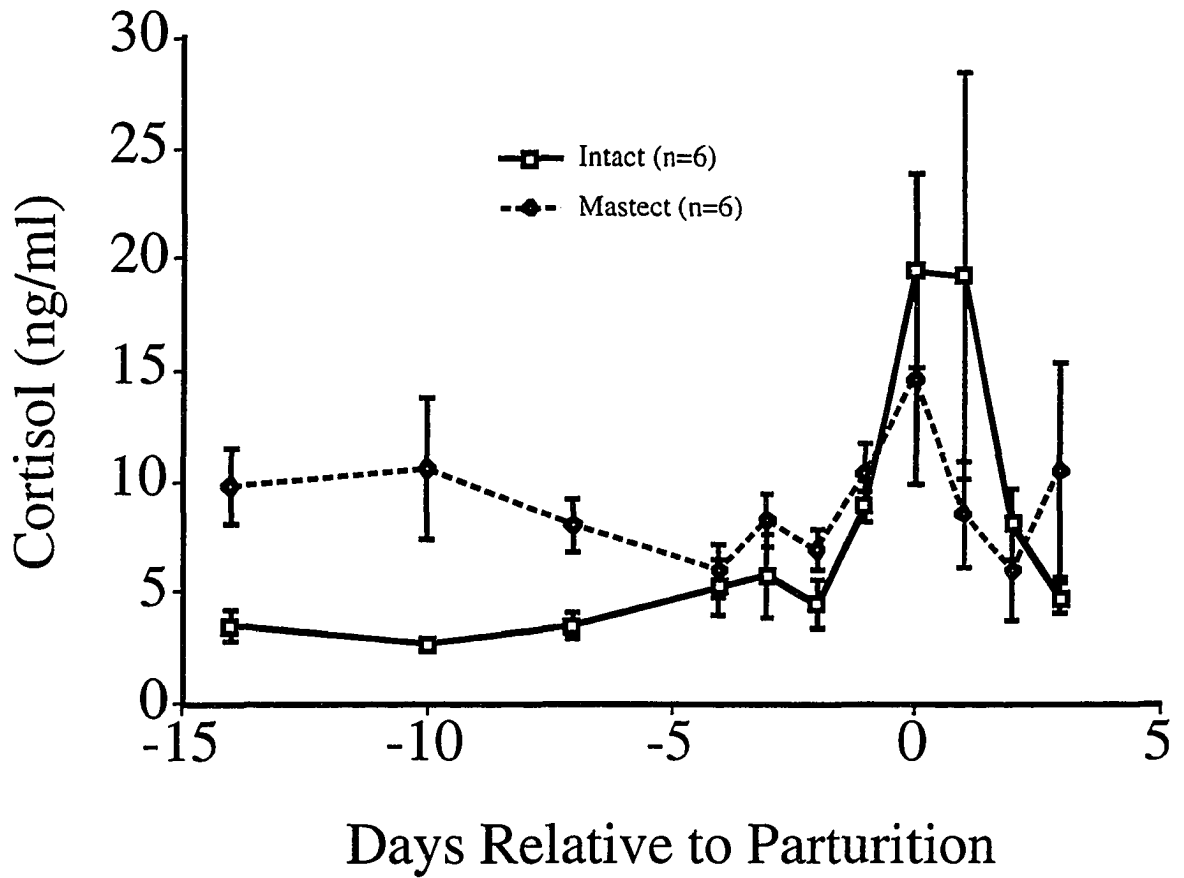
Figure 4. Plasma Cortisol level in intact and mastectomized cows during periparturient period.

GENERAL CONCLUSIONS

Numerous studies have demonstrated a decline in immune cell function during the periparturient period. Our first hypothesis was "Periparturient immunosuppression is closely associated with changes in immune cell populations." In an earlier study in which cows were sampled at weekly intervals, changes in the percentage of CD8 positive cells were not detected, and CD4 positive cells in PBMC exhibited an increase following parturition. Other researchers showed decreases in T cell population at parturition were associated with decreased immune function. However, it was not known exactly when and what changes occur during the periparturient period. Using 8 multiparous Jersey cows with frequent sampling during the 2 wk before and after parturition, we were able to demonstrate that the percentages of CD3, CD4, and γδ T-cell receptor positive cells declined significantly before parturition and reached a nadir at parturition. The nadirs of each subpopulation represented about a 25% decline in the proportion of each subset in the circulation. These percentages then gradually returned to prepartum values during the first 2 wk of lactation. These data are compatible with the hypothesis that declining T-cell populations may contribute to the immunosuppression reported for dairy cows at calving (First paper) since the pattern of change in T-cell populations was similar to the reported changes in PBMC and neutrophil functions. Whether the cell population changes are the cause or the effect of periparturient immune suppression remains unknown.

Our next hypothesis was "Milk production plays an important role in this periparturient immunosuppression both in PBMC and neutrophils". Cows during the periparturient period are exposed to tremendous changes due to induction of parturition and onset of lactation. Both of these may cause immunosuppression. In order to define the effect of the mammary gland and colostrum synthesis on periparturient immunosuppression, we used 10

mastectomized and 8 intact multiparous Jersey cows. First we examined the changes in immune cell population using a bovine specific monoclonal antibodies against T cell, B cell, and monocytes with frequent samplings from -4 to 4 wk after parturition. We found that most changes in immune cell population during periparturient period was due to the presence of the mammary gland, and thus colostrum production. Intact cows showed a significant decline in all T cell subset populations (CD3, CD4, CD8 and γδ-T cell receptor positive cells) while the monocyte percentage increased significantly at parturition. These changes were significantly different from those seen in mastectomized cows except for the CD8 positive cells (Second paper). Mastectomy eliminated almost all changes in leukocyte subsets seen at the time of parturition. These changes are well correlated to the changes in PBMC function. The decline in in vitro production of IFN-γ and IgM at parturition was seen in intact cows whereas mastectomized cows did not show a significant change during the sampling period (Appendix).

We also examined the expression of adhesion molecules (β2-integrins and L-selectin) and myeloperoxidase activity in neutrophils using the same cows as used for the second paper. This study was intended to assess the effect of lactation and mammary gland on the neutrophil function, such as, migration (adhesion molecule expression), ingestion, and killing of invaders (myeloperoxidase activity). Expression of β2-integrins in intact cows was highest at parturition. Expression of β2-integrins was greater in intact cows than in mastectomized cows throughout the study. L-selectin expression exhibited a sudden decrease at parturition with recovery within a day after parturition in both intact and mastectomized cows. The ability of neutrophils to kill microbes as assessed by neutrophil myeloperoxidase activity decreased before parturition in both groups. While there was a quick recovery of neutrophil myeloperoxidase activity in mastectomized cows, there was no recovery in intact cows after parturition throughout the study which lasted until d 20 post partum. Milk production seems

to exacerbate the duration of periparturient immunosuppression, especially with regard to neutrophil myeloperoxidase activity (Third paper).

Then what was the real cause of this periparturient immunosuppression in the presence of mammary gland? It has been speculated that periparturient immunosuppression in dairy cows is caused by high level of steroid hormones and/or negative energy balance associated with parturition and colostrum synthesis. Our mastectomized cow studies showed that mastectomy eliminates almost all immunological changes. We wondered how steroid hormones affect these changes. In order to see the effect of colostrum production and mammogenesis on steroid hormone profiles in association with immune function, we used 6 intact and 6 mastectomized multiparous Jersey cows. We found that mastectomized cows show similar (progesterone, cortisol, and estradiol) or higher levels (estrone) of steroid hormones. These data suggest that high levels of steroid hormones do not seem to be responsible for the further impairment of immune function especially in PBMC seen in periparturient dairy cows, however high level of estrogens and cortisol may have certain effects on myeloperoxidase activity and L-selectin expression in neutrophils (Fourth paper).

Although we could demonstrate that a decline in T cell subsets is associated with impaired immune cell function during the periparturient period, we are not sure why T cell subsets decrease. They may migrate to tissues and stay there around parturition, since there is a recovery in T cell population after parturition. Or, it may be due to cell death.

Presence of the mammary gland plays an important role in the periparturient immunosuppression, but what factors of milk production contribute to this immunosuppression remains unknown. We are still studying the nutritional effects on periparturient immunosuppression, especially the effects of energy, vitamins A, D, and E, and mineral balance. These studies may answer the question of a nutritional effect. As lactation requires other hormones, such as growth hormone and prolactin, we need to know if these hormones which are known to be immunomodulators, may have affected periparturient

immunosuppression. In addition to these factors, there may be the effects of various cytokines, opioids, neurotransmitters, etc. which can modulate immune function. Further studies are needed to elucidate the real cause of periparturient immunosuppression, thus enabling prevention of this immunosuppression.

APPENDIX

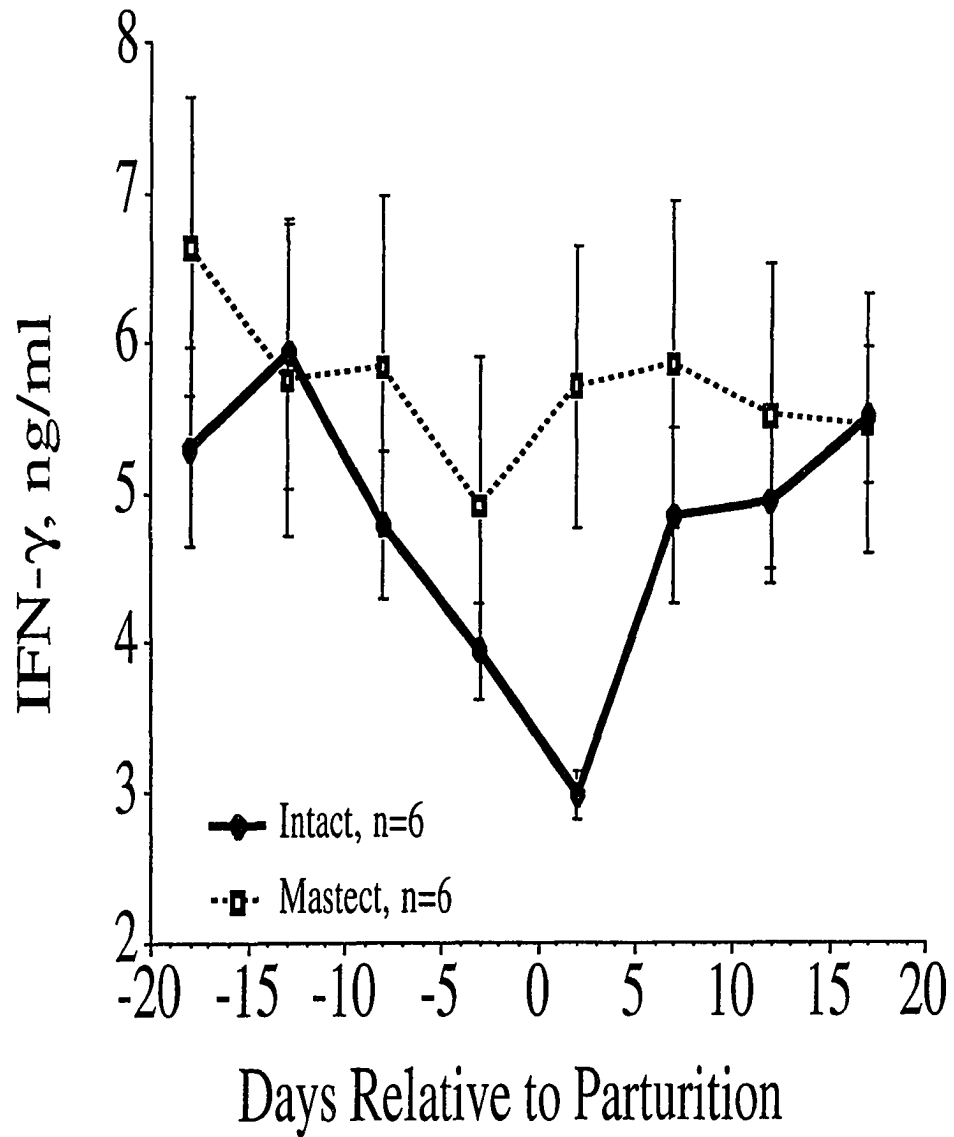Graphs of Immune Function of Peripheral Blood Mononucles Cells
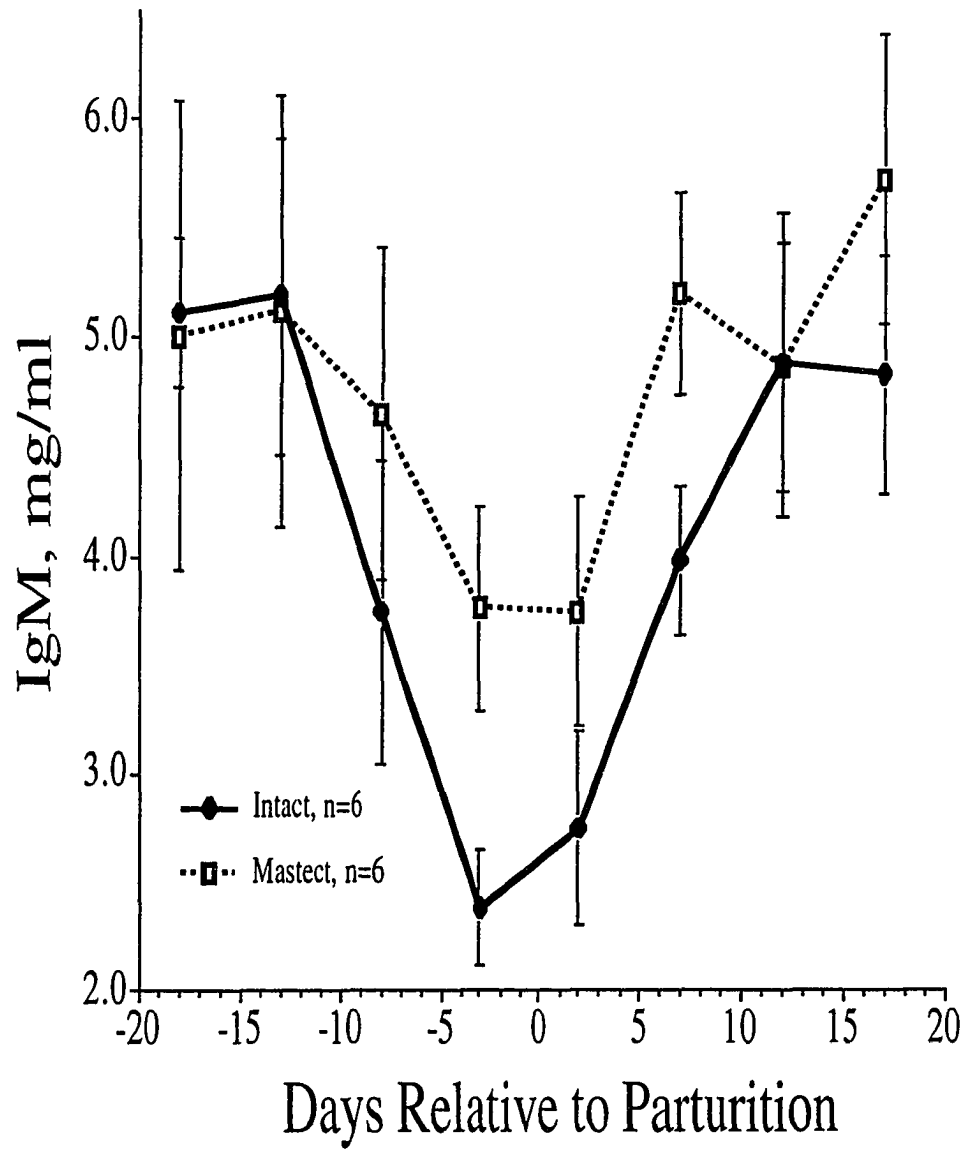
Figure A1. In vitro production of interferon-γ

Figure A2. In vitro production of IgM.

ACKNOWLEDGEMENTS

It has been a dream of mine to study in the US and get a Ph.D. ever since I was in elementary school. Poverty and several circumstances did not allow me to realize this dream for a long time. When I turned age 40, I wondered if I can die happily. I want to be able to say as in Frank Sinatra's song, "I did it my way.". " Regrets? I have a few but I did everything I wanted." My life is only once, I cannot be born again. I decided to come to the US, quitting my job as a manager in a research laboratory although I had a challenging job under the severe gender discrimination in my country. In 1993, I finally decided to apply for ISU. My "American brother" Dr. Keith Whigham helped me with this application. Thanks to his great help and the generosity of Dr. Engen and Dr. Ahrens, I was accepted as a Ph.D. student of Veterinary Physiology and Pharmacology and I came to Ames on August 12, 1993.

When I applied for ISU, I wondered what special area I should study. Since my parents are dairy farmers, I have always had a great interest in dairy cows, especially how to save the lives of dairy cows from diseases. Many researchers are interested in infectious diseases, but I thought, if cows have healthy bodies, they would not suffer from infectious diseases. Resistance to metabolic diseases as a result of improved nutrition, may be a factor in preventing infectious diseases. I decided to study metabolic diseases in dairy cattle. In the list of professors and their research interests, I found that Dr. Jesse Goff is the best as my major professor. I could not be Dr. Goff's student at the beginning. Since study at ISU is only one chance for me to do my favorite research, I could not give up trying to be his student. After several months' struggling in my mind, I visited Dr. Goff on March 8, 1994 for the first time. When I saw the plate which read "Metabolic Diseases and Immunology", I was very excited. "Yes, this is the place where I want to do my research!" Finally, I started my research training at NADC from the beginning of August, 1994. I had to take a long, roundabout way to come

here. I tried to give up the idea of getting a Ph.D. in the US many times. I told myself "I have no sense as a researcher.", but I could not give up my dream. My research in NADC has not been easy but I have been very happy being with my lovely cows and I had kind support of many staff. Thanks to the great idea and the generosity of Dr. Jesse Goff, my research sense was awakened. Dr. Goff has always been pushing (?) and testing me, but at the same time he gave me a lot of chances to be a good researcher. I really appreciate him for giving me a chance to enjoy my research life here. Thanks to his support, I have presented my findings at the scientific meetings 7 times so far.
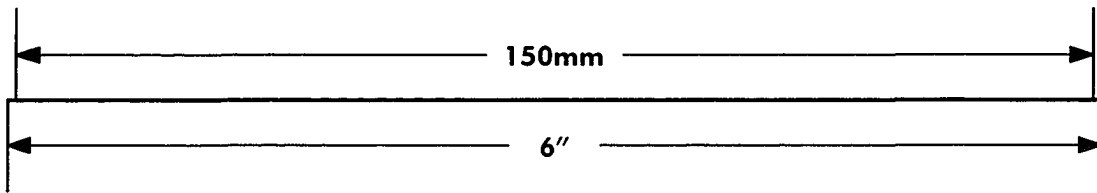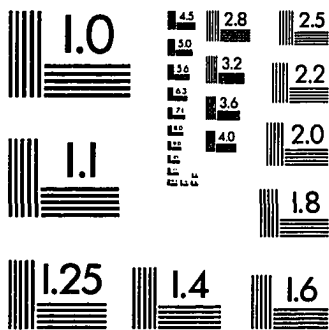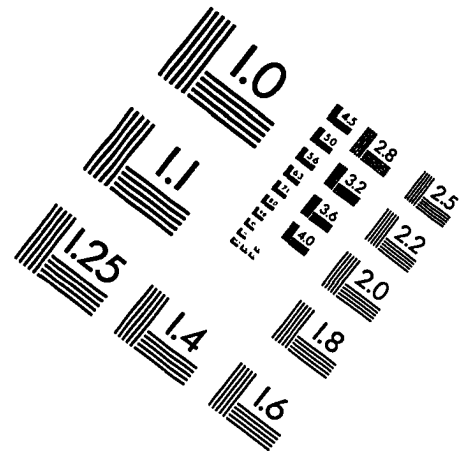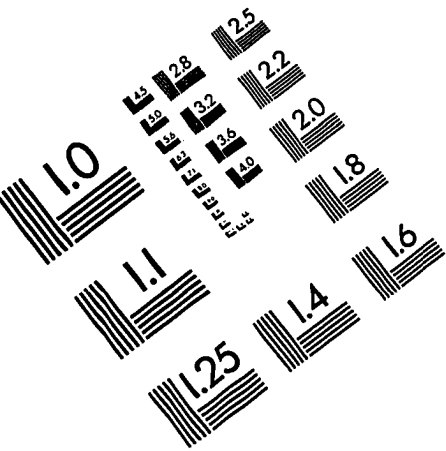
The next important person is Dr. Marcus Kehrli, Jr. He taught me many things not only about immunology but also how a computer works, and the methodology necessary for my research. Dr. James Harp and Dr. Brian Nonnecke have given me a good suggestions for my research and they have helped me a lot. Dr. Ronald Horst is helping me to have good research conditions. Although Dr. Timothy Reinhardt and Dr. Mary Jo Schmerr are not my committee members, they have been very supportive of my research.

Many people supported my research, especially the animal care takers, Norman Tjelmeland, Creig Caruth, Bruce Grey took wonderful care of my animals and all the MDI technicians helped me a lot. Special appreciation goes to Bruce Pesch who taught me flow cytometry. Arlen Anderson is helping me with a lot of things for my research. I am so sorry but I cannot name all people in NADC who were so kind. I would like to extend my appreciation to all MDI students (current and past) who shared the pains and joys of work.

Finally I would like to extend my appreciation to all of my friends in Ames and Japan who encouraged me to continue my research. Kate Mason, my best friend here in Ames, has been helping me with many aspects including the proofreading of almost all of my papers. John Mason has been teaching me American humor and how to pronounce English properly. Linda and Jere Maddux, and all members of Town and College Toastmasters Club have given me a spice in my life in Ames. Thanks to my good friends in Japan, especially Sachiko Shimura,

who has been sending me Japanese foods and encouraging me from Japan, so I would not need to miss Japan so much. I also have to appreciate my parents, sister and brother who had to suffer from having a strange family member like me.

IMAGE EVALUATION
TEST TARGET (QA-3)

1.0

1.1

1.25   1.4   1.6

2.8   2.5

3.2   2.2

3.6   2.0

4.0   1.8

←——————— 150mm ———————→

←——————— 6" ———————→