NEGATIVE ENERGY BALANCE AND UNCOUPLING OF THE GROWTH HORMONE/ INSULIN-LIKE GROWTH FACTOR I AXIS IN CATTLE: IMPLICATIONS FOR IMMUNOCOMPETENCE

By

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ABSTRACT

Negative energy balance and uncoupling of the growth hormone/ insulin-like growth factor I axis in cattle: Implications for immunocompetence

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Dairy cows are immunosuppressed around the time of parturition, which may be due to negative energy balance (NEB). One mechanism by which this may occur is by uncoupling of the growth hormone (GH) / insulin-like growth factor I (IGF-I) axis by NEB. During NEB in cattle, the GH/IGF-I axis is uncoupled via down-regulation of GH receptors in the liver. The hypothesis of this study is that NEB also decreases leukocyte expression of GH and (or) IGF-I receptors, thereby contributing to impaired immunocompetence. The objective of this study was to develop a steer model of bovine NEB to monitor bovine leukocyte expression of these receptors as well as key adhesion (CD62L, CD11b, and CD18) and antigen-presenting (MHC class I and II) molecules. Holstein steers (n=16) were assigned randomly to two groups and fed diets that resulted in positive energy balance (PEB) or NEB (210% and 60% of maintenance requirements, respectively). NEB decreased the percentage of mononuclear leukocytes expressing GH and IGF-I receptors. Expression of these molecules by neutrophils and of other molecules was not affected adversely. Therefore, NEB may regulate GH and IGF-I receptor expression in some circulating leukocytes, but does not appear to adversely affect competence of these cells in terms of gene expression in the absence of infection.

This thesis is dedicated to my parents for never letting me think there was anything I couldn't do and for giving me every opportunity to do it. It is also dedicated to Nick for sticking with me every step of the way with constant love, encouragement, honesty and most of all, patience.

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This thesis would never have happened without the help of many people. First, I would like to thank my major professor, Dr. Mike VandeHaar for giving me the freedom to explore new ideas and do things a little differently.

I owe special thanks to Dr. Jeannie Burton for going above and beyond the call of duty for a committee member by sparking my interest in immunology, for providing opportunities I might have otherwise missed, and of course for her friendship.

I would like to thank the other members of my guidance committee; Drs. Dave Beede and Tom Herdt for valuable input to this thesis and my graduate program.

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I also would like to thank the immunogenetics lab for "adopting" me. Your input and friendship has been invaluable. I would also like to thank Dr. Jeannie Burton, Jennifer Wells, and Mark Jacob for conceptual and graphic design of the neutrophil migration figure used in this thesis. I can't name them all but I must thank the all of the grad students for good company, valuable brainstorming and a beer every now and again. The experience would not have been the same without MSU hockey and PT's.

I also would like to thank the Michigan Soybean Promotion Committee, the Corn Marketing Program of Michigan and the Michigan Agricultural Experiment Station for funding this research and Dr. Wesley Warren (Protiva Unit, Monsanto Inc., St Louis, MO) for his generous donation of the anti-GH receptor antibody.

Lastly, I must thank the people who have seen me through it all; Nick and my parents, Peg and Jerry Perkins. Nick I owe you more than anyone for seeing me through the rough spots, being my best source of honest feedback, and never letting me lose sight of my goal. You have been the love and support that kept me going every step of the way, I couldn't have done it without you! And finally, I would like to thank my parents for encouraging me to pursue my interest in "cow science" and for supporting me, unconditionally, through everything.

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LIST OF ABBREVIATIONS

bST: Recombinant bovine somatotropin

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CD11b: Cluster of differentiation 11b

CD18: Cluster of differentiation 18

CD62L: Cluster of differentiation 62L

DMI: Dry matter intake

FITC: Fluorescein isothiocyanate

FSC: Forward scatter

GH: Growth hormone

IGF-I: Insulin-like growth factor I

IL-1: Interleukin-1

IL-2: Interleukin-2

LSM: Least squares mean

MFI: Mean fluorescence intensity

MHC: Major histocompatability complex

NEB: Negative energy balance

NEFA: Non-esterified fatty acid

PE: Phycoerythrin

PEB: Positive energy balance

PEM: Protein-energy malnutrition

SSC: Side scatter

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INTRODUCTION

The immune system is altered by many physiologic changes including nutritional status. Protein-energy malnutrition (PEM) in humans adversely affects many aspects of the immune system (Chandra, 1997; 1992; 1991; 1973; Chandra and Newberne, 1977; Beisel, 1996; Keith and Jeejeebhoy, 1997; Hoffman-Goetz et al., 1984). This may be via uncoupling of the growth hormone (GH) and insulin-like growth factor I (IGF-I) system that otherwise has immunoenhancing effects (Commens-Keller et al., 1995). In PEM the normal positive relationship of the GH/IGF-I system is severed, and receptors for GH in the liver are down-regulated resulting in elevated serum GH and decreased serum IGF-I (McGuire et al., 1992a; Vicini et al., 1991). This scenario also is observed in periparturient dairy cows, which experience negative energy and protein balance that causes mobilization of body reserves in the form of non-esterified fatty acids (NEFA; Grummer, 1995) and separation of the GH/IGF-I axis (Perkins, 1997). Little research has been published which tests effects of GH/IGF-I uncoupling on the bovine immune system.

Dairy cows are immunosuppressed during the periparturient period (Table 1.2; Lee and Kehrli, 1998; Detilleux et al., 1995). During this period, expression of the vital adhesion molecules CD62L and CD18 by bovine blood neutrophils (Lee and Kehrli, 1998), ability of neutrophils to kill bacteria (Nagahata et al., 1988), and lymphocyte function are all decreased (Kehrli et al., 1989a). The percentages of mononuclear leukocytes that express MHC class II molecules are highest before calving but decrease immediately after parturition (Van Kampen

and Mallard, 1997). Furthermore, a study of cows on commercial dairy farms found that animals with high serum concentrations of NEFA around calving and early lactation had an increased incidence of mastitis and retained placenta postpartum (Dyk et al., 1995). Together, these observations suggest that there is a relationship between negative energy balance (NEB) and disease incidence that may be mediated by effects of uncoupling of the GH/IGF-I axis on immunocompetence. If true, this would infer altered expression of receptors for these hormones, not only in the liver but also by immune cells, resulting in decreased immunocompetence and increased disease susceptibility during NEB.

Although the relationship between energy nutrition and immune function is complex, I have initiated studies to identify several mechanisms by which NEB may alter immunocompetence in cattle. Understanding the interaction between GH, IGF-I and the immune system, would not only benefit the dairy industry but could have profound implications for improving human health via nutritional means.

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Chapter 1

LITERATURE REVIEW

Negative energy balance uncouples the GH/IGF-I axis

Growth hormone (GH) plays a key role in regulating the insulin-like growth factor (IGF) system. When animals are given exogenous GH, circulating concentrations of IGF-I are increased (Cohick et al., 1989). It originally was thought that the IGF system functioned solely via endocrine signals and that GH caused the liver to produce IGF-I, which then traveled in the circulation to various target tissues. However, we now know that there are many tissues that contain IGF-I mRNA, including immune cells, but in lower abundance than is found in the liver (Clemmons and Underwood, 1991; McGuire et al., 1992b). Thus IGF-I may act via endocrine, autocrine and/or paracrine control.

The effects of GH on the IGF system are influenced by nutritional status (Figure 1.1; McGuire et al., 1992a; Vicini et al., 1991; Underwood et al., 1994; Yung et al., 1996). In a study by McGuire et al. (1995), dairy cows were subjected to two dietary treatments. In one they were fed 120% of NRC requirements and the other they were deprived of feed for 2 days. In both treatments the cows were given a challenge of exogenous GH. The fed cows had an increase in the concentration of circulating IGF-I following the GH administration, but the fasted cows did not (McGuire et al., 1995). Elsasser et al. (1989) also found that undernutrition in steers attenuated the response of IGF-I to GH. This nutritional modulation of the effect of GH on the IGF system may

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be a method by which the animal protects itself during starvation. The animal reduces the use of nutrients for production, providing metabolic fuel for maintenance during NEB (McGuire et al., 1995).

The separation of the GH/IGF-I axis during malnutrition is due most likely to down-regulation of GH receptors by the liver (Figure 1.1). Expression of GH receptor mRNA and GH binding sites in liver of rats fed low-protein diets was significantly lower ($P < 0.01$) than that of rats fed control diets (VandeHaar et al., 1991). In a study comparing high and low planes of nutrition in lambs, GH binding in liver membranes also was lower ($P < 0.01$) in lambs fed a low protein diet (Bass et al., 1991). In cattle, steers fed at 1% of body weight had lower affinity hepatic GH binding sites than those fed at 3% of body weight (Breier et al., 1988). Little research has been published that has examined the impact of malnutrition on expression of GH receptors in other cell types.

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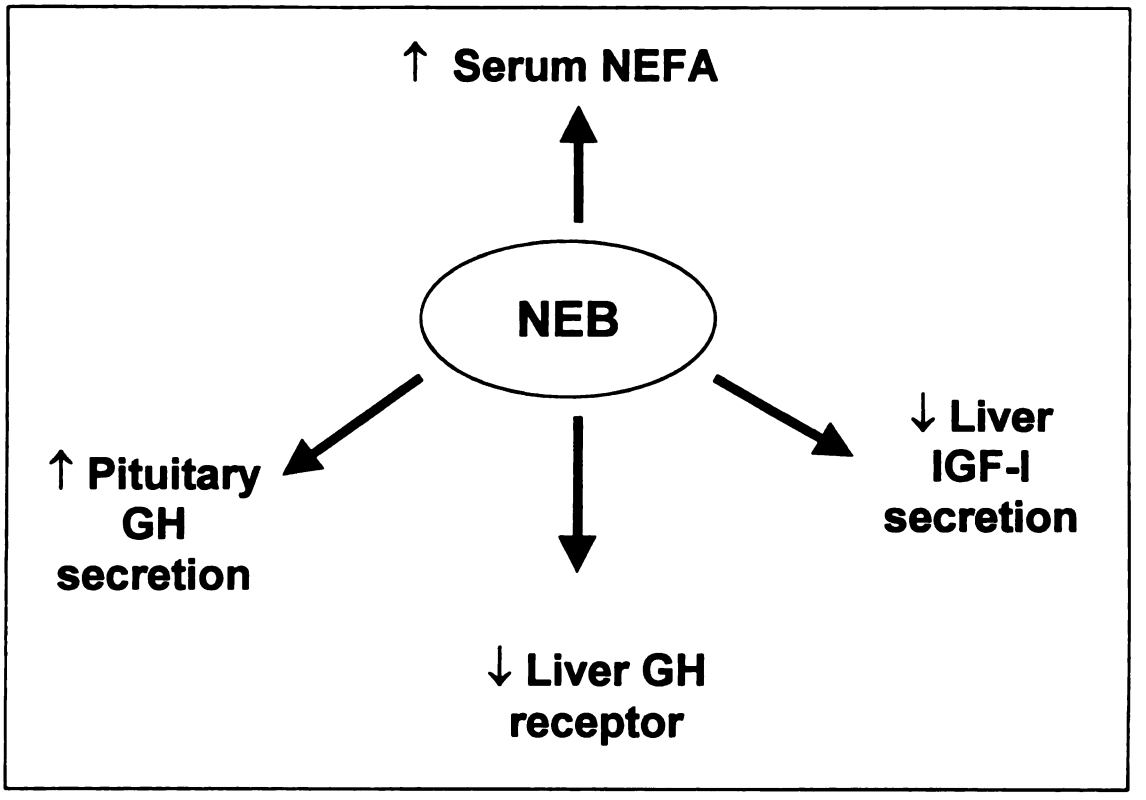

Figure 1.1. Uncoupling of the growth hormone/insulin-like growth factor I axis in cattle.

Negative energy balance and the periparturient dairy cow

A. Metabolites and Hormones in the Periparturient Period

Dairy cows face an enormous metabolic challenge in making the transition from late pregnancy to lactation, which may contribute to decreased immunocompetence at this time. This periparturient period is defined as approximately 3 weeks prior to calving through 3 weeks after calving (Grummer, 1995). At the onset of lactation many adaptations must occur to meet the metabolic demands of the mammary gland (Bauman and Currie, 1980). Some of the metabolic changes that the dairy cow undergoes are shown in Table 1.1.

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There are also other changes during the periparturient period that create a challenge for the animal. Dry matter intake (DMI) decreases gradually during the last 3 weeks prepartum, especially in the last week before parturition (Grummer, 1995). In the typical dairy cow, DMI decreases as much as 30% prior to calving, imposing NEB (Grummer, 1995). In high producing dairy cows, voluntary intake does not achieve an amount sufficient to meet the cows' protein and energy requirements until about 60 days postpartum (Bauman and Currie, 1980).

Table 1.1. Partial list of metabolic changes associated with the onset of lactation in dairy cows.

Physiological Function	Metabolic Change	Tissues Involved
Milk Synthesis	Increased use of nutrients	Mammary
Lipid Metabolism	Increased lipolysis Decreased lipogenesis	Adipose tissue
Glucose Metabolism	Increased gluconeogenesis Increased glycogenolysis	Liver
	Decreased use of glucose and increased use of lipid as energy source	Body tissues in general
Protein Metabolism	Mobilization of protein reserves	Muscle and other body tissue
Mineral Metabolism	Increased absorption and mobilization of calcium	Kidney, liver, gut, and bone

Adapted from Bauman and Currie, 1980

Metabolites and hormones also undergo changes during the periparturient period. One of the major changes occurs in adipose tissue where there is decreased nutrient uptake and increased mobilization of lipid reserves (Table

1.1; Bauman and Currie, 1980). Circulating concentration of NEFA increases during the peripartum period (Grummer, 1995). This is due to mobilization of adipose tissue triglyceride stores during the transition from pregnancy to lactation (Bell and Bauman, 1996). The circulating concentration of NEFA almost doubles from 17 days prepartum until 2 days prepartum. NEFA concentrations reach a peak the day of or the day after parturition (Grummer, 1995). The circulating concentration of NEFA declines after parturition but remains higher than during the dry period as long as the cow is in NEB (Bell and Bauman, 1996). A study conducted on commercial dairy farms found that cows with high serum NEFA prepartum had an increased incidence of mastitis and retained placenta postpartum (Dyk et al., 1995), suggesting a link between NEB and disease susceptibility.

Plasma insulin decreases approximately a week before parturition and remains low during the first few weeks after parturition. This, together with an increase in GH, allows for nutrients such as glucose to be partitioned to the mammary tissue in preparation for the onset of lactation (Grummer, 1995). It should be noted that the concentration of endogenous GH is highest during the periparturient period, especially during the 3 weeks following calving (Grummer, 1995). GH is involved directly in modifying adipose metabolism to allow the use of these energy reserves especially in the periparturient period (Bell and Bauman, 1996). GH also exhibits homeorhetic control by directing absorbed nutrients toward growth and lactation in well-fed animals (Simmons et al., 1994).

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This seems to involve an indirect mechanism where effects are mediated by an intact (i.e., GH-responsive) IGF system (Bauman and Vernon, 1993).

The IGF system also undergoes significant changes during the transition period. The circulating concentration of IGF-I decreases at about 3 weeks prepartum (Simmons et al., 1994; Vega et al., 1991; VandeHaar et al., 1999). Vega et al. (1991) found that although the circulating concentration of IGF-I showed a constant decline until parturition, the greatest change occurs between 4 days before and 3 days after parturition, with a 70% reduction in IGF-I. Because the concentration of IGF-I in serum is low, and the concentration of GH in serum is high, the GH/IGF-I axis is said to be uncoupled during NEB.

B. The GH/IGF-I axis in the periparturient dairy cow

In a study examining the GH/IGF-I axis during the periparturient period, cows were given challenges of exogenous GH (bST) throughout the periparturient period. There was a change in the effect of bST on the circulating concentration of IGF-I through the transition from late pregnancy to early lactation ($P < 0.05$; Perkins, 1997). The circulating concentration of IGF-I increased 123% after the bST challenge 30 days prepartum. This response was attenuated gradually until parturition when, at day 2 postpartum, there was no response in circulating concentration of IGF-I after administration of bST (Perkins, 1997). Circulating concentration of IGF-I also was at its lowest at day 2 postpartum with a mean ± SEM of 79 ± 7 ng/ml (Figure 1.2; Perkins, 1997).

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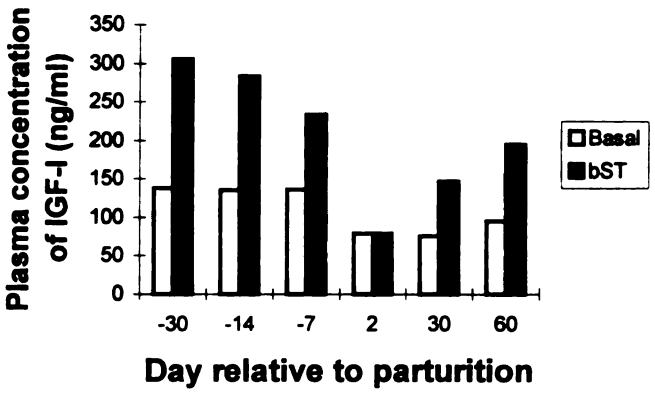

Figure 1.2. Response of IGF-I to a 3-d challenge of 45 mg exogenous bST in Holstein dairy cows at different stages of the transition period from late pregnancy to early lactation. Pooled SEM was 10 ng/ml (Perkins, 1997).

Similarly, Simmons et al. (1994) found that the GH/IGF-I axis was still intact 1 week before parturition as indicated by a rise in IGF-I following GH administration. However, at 1 day before parturition the same dose of GH did not cause an increase in serum IGF-I, indicating uncoupling of the GH/IGF-I axis.

Separation of the GH/IGF-I axis during the periparturient period is suggested to be due to NEB via down-regulation of GH receptors in the liver and the need to partition nutrients to the mammary gland for lactation. Little research has been published that examined expression of GH receptors on other cell types during NEB or during the periparturient period.

Disease susceptibility of periparturient dairy cows may be linked with NEB

A. Metabolic disorders in the periparturient dairy cow

In dairy cows in early lactation, the demand of the mammary gland for nutrients often exceeds the amount available. This leads to NEB, increased fat mobilization and increased hepatic ketogenesis. There are relatively few studies

that have examined the relationship between metabolism and immuno-competence in cattle. One such study found that the ability of bovine lymphocytes to respond to the mitogen phytohemagglutinin (PHA) was reduced when the lymphocytes were preincubated with β-hydroxybutyrate or acetoacteate (Targowski and Klucinski, 1983), which are found in the circulation of cows in severe NEB. This suggests that in the presence of these fatty acids, lymphocytes are not able to perform optimally. High liver fat in dairy cows is associated with a reduction in the number of peripheral white blood cells and lower milk somatic cell counts suggesting dysfunctional leukocyte migration (Reid et al., 1983). In addition, Ropstad et al. (1989) found a positive relationship between energy balance and the lymphocyte response to mitogens. Similarly, a study by Cai et al. (1994) found a positive relationship between decreased neutrophil function and periparturient disorders, such as retained placenta, metritis, and mastitis. Combined, these studies suggest that there is a relationship between NEB and decreased immunocompetence in cattle.

B. The periparturient dairy cow and susceptibility to infectious disease

Dairy cows are immunosuppressed in the periparturient period (Table 1.2; Lee and Kehrli, 1998; Detilleux et al., 1995). This is also the time that the mammary gland is markedly susceptible to new intramammary infections (Oliver and Mitchell, 1983). One possible explanation for increased susceptibility to disease could be NEB at this time as indicated by elevated serum NEFA. A study on commercial dairy farms found that cows with high NEFA before calving had an increased incidence of mastitis and retained placenta after calving (Dyk et

al, 1995). Many different aspects of the immune system are compromised at this time (Table 1.2). Human immunocompetence during NEB also is impaired (Chandra, 1997).

Table 1.2. Summary of leukocyte dysfunctions in periparturient dairy cows.

Leukocyte Type	Dysfunction	Reference
Neutrophil	↑ Blood neutrophil counts	Detilleux et al., 1995 Kehrli et al., 1989b Newbould, 1976 Preisler et al., 1999
	↑ Random migration *in vitro*	Kehrli et al., 1989b Nagahata et al., 1988
	↑ Bacterial ingestion by phagocytosis *in vitro*	Kehrli et al., 1989b Kehrli and Goff, 1989 Saad et al., 1989
	↓ Oxidative metabolism *in vitro*	Kehrli and Goff, 1989 Detilleux et a., 1995 Kehrli et al., 1989b
	↓ Neutrophil chemotaxis *in vitro*	Nagahata et al., 1988
	↓ Antibody dependent cell-mediated cytotoxicity *in vitro*	Cai et al., 1994 Kehrli et al., 1989b
	↓ CD62L and CD18 expression *in vivo*	Lee and Kehrli, 1998
Monocyte/ Macrophage	NA	NA
Lymphocyte	↓ Lymphocyte blastogenesis in response to PHA, PHAP, ConA and PWM *in vitro*	Detilleux et al., 1995 Saad et al., 1989 Kehrli et al., 1989a Kehrli and Goff, 1989
	↓ Blood lymphocyte counts	Kehrli et al., 1989a
	Changes in T-lymphocyte subpopulations *in vivo*	Van Kampen and Mallard, 1997 Kimura et al., 1999
	↓ Antibody producing activity *in vitro*	Nagahata et al., 1992

C. Human PEM-disease associations as a model

There are many studies that demonstrate a link between protein-energy malnutrition (PEM) and impaired immunocompetence. PEM is associated with significant impairment of cell-mediated immunity, phagocyte function, complement concentration and activity, secretory immunoglobulin A antibody concentrations, and cytokine production (Chandra, 1997).

Generalized PEM in humans causes widespread atrophy of lymphoid tissues, especially in children. The thymus, tonsils, spleen and lymph nodes are all affected (Chandra and Newberne, 1977; Beisel, 1996). There is also a decrease in the number of mature and fully differentiated circulating T-lymphocytes and eosinophils (Chandra, 1972; 1997; Beisel, 1996). Flow cytometric techniques and monoclonal antibodies demonstrated a decrease in the proportion of $CD4^+$ T-lymphocytes in PEM (Chandra, 1992). Conversely, B-lymphocyte numbers and functions seem to be maintained in PEM although new antibody responses to T-cell dependent antigens, and antibody affinity for antigen are impaired (Beisel, 1996; Chandra, 1972; 1991). Non-specific immune responses also are affected by PEM. The ability of neutrophils and monocytes to phagocytose and kill bacteria is impaired (Salimonu et al., 1982; Chandra, 1991). This is attributed to reduced activity of several key metabolic pathways that produce reactive oxygen species needed to kill bacteria (Chandra, 1992). PEM also is associated with decreased concentrations of almost all complement components (Chandra, 1973). Finally, the ability of the individual to produce

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cytokines and the response of T-lymphocytes to cytokines (IL-1, IL-2) is impaired (Keith and Jeejeebhoy, 1997, Hoffman-Goetz et al., 1984).

Immunological dysfunctions associated with malnutrition have been termed "nutritionally acquired immune deficiency syndromes" or NAIDS. The combination of NAIDS and common childhood infections is the leading cause of human mortality (Beisel, 1996), providing anecdotal evidence that malnourished humans are more susceptible to infectious disease. I speculate in this thesis that malnourished cattle also may be immune deficient and more susceptible to infectious disease.

Resistance to infectious diseases depends on host physical barriers and immunocompetency

A. Immunocompetency

The immune system is designed to protect the body from those substances or organisms that are recognized as 'non-self,' and to respond to these foreign antigens in both innate and highly specific ways. To effectively combat foreign antigens, like the ones that cause peripartum diseases in dairy cows, the immune system utilizes a myriad of cell types and secretory factors (interleukins; IL). These act to activate phagocytes and to expand lines of antigen-specific lymphocytes that will seek out and destroy antigen. However, before a specific response can be mounted, the antigen must be able to penetrate some or all of the animal's natural barriers to infection.

1. Physical and Biochemical Barriers

Numerous anatomic structures and their secretory components prevent entry of pathogens into peripheral tissues and organs. These include barriers

such as the skin, hair, and mucous membranes. Most infectious agents gain access to the mammary gland of cattle by entering the teat canal. A physical barrier of keratin acts as a "plug" and also can exhibit bacteriostatic and/or bactericidal effects (Senft and Neudecker, 1991) that protect the teat canal. If the keratin barrier is overcome or unable to keep the bacteria out, bacteria enter the teat cistern and will encounter the second immunological line of defense, phagocytic neutrophils of the innate immune system (Paape and Capuco, 1997).

2. Innate Immunity

The innate immune system always is present and can respond immediately, but with limited specificity, to extracellular pathogens. Once foreign antigen enters the mammary gland, the innate immune system functions using soluble proteins in conjunction with phagocytic and killing mechanisms of neutrophils and monocytes (macrophages when differentiated in the tissue). The neutrophil is especially important in protecting the mammary gland from infectious disease in dairy cattle and it is the primary leukocyte responsible for clearing bacterial infections in the mammary gland (Burvenich et al., 1994). If these phagocytic leukocytes are unable to enter the gland or are unable to clear the infection, the adaptive immune system must be called upon to aid in clearing the gland of infection.

3. Adaptive Immunity

Immunity that is dependent on the body's ability to specifically recognize and selectively eliminate foreign antigens is called adaptive immunity (Kuby,

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1997). There are two main branches of adaptive immunity: humoral immunity and cell-mediated immunity.

Humoral immunity is mediated by soluble antibodies that are produced by antigen-activated B-lymphocytes. The main function of antibodies is to bind extracellular antigen and promote opsonization for easier and more targeted uptake by phagocytic cells of the innate immune system (Figure 1.3; Kuby, 1997). Antigen-activated T-lymphocytes regulate both cell-mediated and humoral immunity. T-lymphocytes express a unique antigen binding receptor (T-cell receptor). This receptor recognizes only antigen that is associated with cell membrane proteins called major histocompatibility complex (MHC) molecules. If MHC expression is impaired, it will be impossible for T-lymphocytes to recognize and respond to antigen, resulting in decreased competence of both the adaptive and the innate arms of the immune system, and possibly increased susceptibility to disease. Therefore, not only is MHC expression vital to immune function, but it is also a valuable indicator of adaptive immunocompetence.

All T-lymphocytes express T-cell receptors but they can be differentiated into a number of subsets by their unique expression of molecules called clusters of differentiation (CD). For example, T-lymphocytes that express CD_4 only recognize foreign antigen associated with class II MHC molecules and T-lymphocytes expressing CD_8 only recognize foreign antigen associated with class I MHC molecules (Kuby, 1997). Expression of CD_4 or CD_8 also defines the function of the T lymphocyte. CD_4+ T lymphocytes generally function as T helper (T_H1 or T_H2) cells whereas CD_8+ T lymphocytes function as T cytotoxic (T_C) cells.

T_H cells connect the acquired and innate arms of the immune system (Figure 1.3; Kuby, 1997). T_C cells are activated by interaction with antigen-class I MHC complex on the surface of altered self-cells. Once activated the T_C cell acquires cytotoxic activity and eliminates altered self-cells by cytolysis (Figure 1.3; Kuby, 1997). These interactions of the innate and adaptive immune system are vital for protecting the mammary gland from invading bacteria and generally are considered part of the inflammatory response. Perhaps the most important interaction in the mammary gland is opsonization of invading pathogens by antibodies produced by B-lymphocytes. This aids the phagocytic neutrophils in recognizing and ingesting the pathogen (Figure 1.3).

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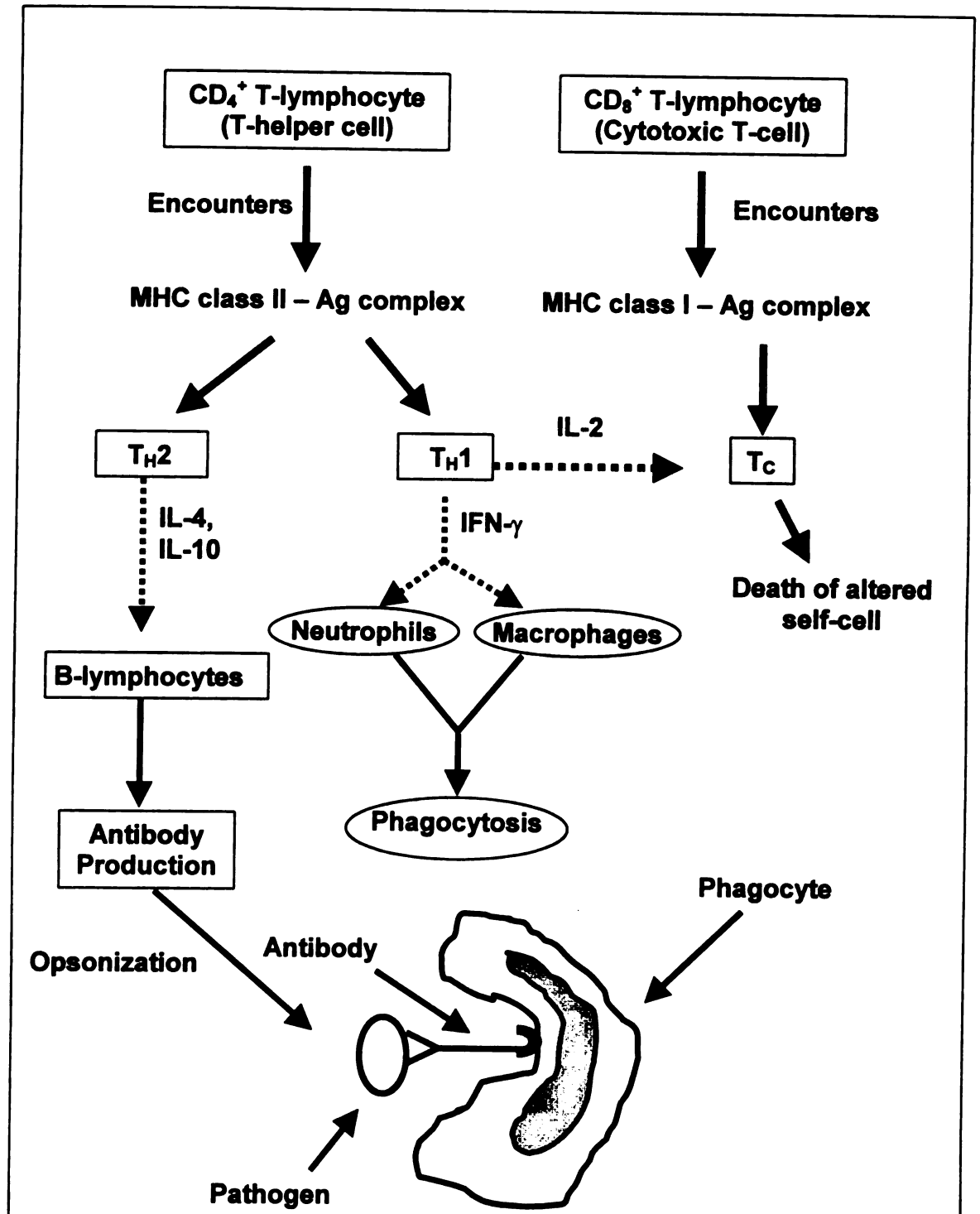


Figure 1.3. T-lymphocytes encounter antigen bound to MHC and differentiate into T-helper (T_H) and T-cytotoxic (T_C) lymphocytes that connect the adaptive (rectangles) and innate (circles) immune systems

B. Inflammation

The two branches of the immune system are by no means independent. In many cases they interact to mount a full immune response. One example of this is the inflammatory response. During inflammation, leukocytes are specifically recruited into infected tissues such as the mammary gland. To reach the infected site, leukocytes must "sense" the presence of infecting pathogens, attach to the endothelial cells lining the capillaries in the inflamed region, migrate between the endothelial cells and into the tissue and destroy the invading pathogens. Several molecular mechanisms have evolved to accomplish this including expression of various cellular adhesion molecules on leukocytes and endothelial cells. The adhesion process of neutrophils can be described as a cascade of four molecular steps (Figure 1.4); tethering (A), triggering (B), strong adhesion (C) and migration (D).

Tethering is mediated by the selectins. This family of three lectin-like membrane glycoproteins bind specific carbohydrates on the glycocalyx of endothelial cells and facilitate leukocyte rolling or margination (Adams and Shaw, 1994). L-selectin (CD62L) is constitutively expressed on most leukocytes, whereas E-selectin is expressed by endothelial cells in response to cytokine activation, and P-selectin is expressed by endothelial cells and platelets (Adams and Shaw, 1994). L-selectin is found on the tips of leukocyte microvilli, which are the first contact points with the endothelium. The adhesions created by the selectins along with the shear force of blood flow are strong enough to induce slowing down via rolling along the vessel wall, but do not stop leukocyte

movement completely (Figure 1.4A). Rolling allows the leukocytes to "survey" the area for the presence of triggering factors that would cause the adhesion cascade to continue. The population of neutrophils involved in this process is referred to as the marginating pool of neutrophils. In the absence of these factors, the leukocyte disengages from the endothelium and is washed away by blood flow to become the circulating pool of neutrophils (Adams and Shaw, 1994).

Another family of adhesion molecules called integrins mediates strong adhesion of leukocytes to the endothelium. Integrins are a family of glycoproteins that contain an α and a β subunit. These integrins must be activated to bind effectively to receptors on the endothelium. Once they are activated by a triggering step (Figure 1.4B), they promote strong adherence that, in combination with shedding of L-selectin, can stop the rolling leukocyte (Figure 1.4C; Kuby, 1997; Adams and Shaw, 1994). One of the most potent trigger factors in cattle is platelet-activating factor (PAF), a phospholipid produced by endothelial cells in response to histamine, thrombin or leukotrienes (Adams and Shaw, 1994). The integrins can be grouped based on their β subunits, which can be either a $\beta 1$ or a $\beta 2$ subunit. For a review of the $\beta 1$ integrins refer to Adams and Shaw (1994). The $\beta 2$ integrins are of particular interest in dairy cattle because they mediate neutrophil migration into infected peripheral tissue (Figure 1.4C and D).

The $\beta 2$ integrins share a common β chain, CD18, which is a 95-kDa glycoprotein that non-covalently couples to CD11 (α) chains to form active

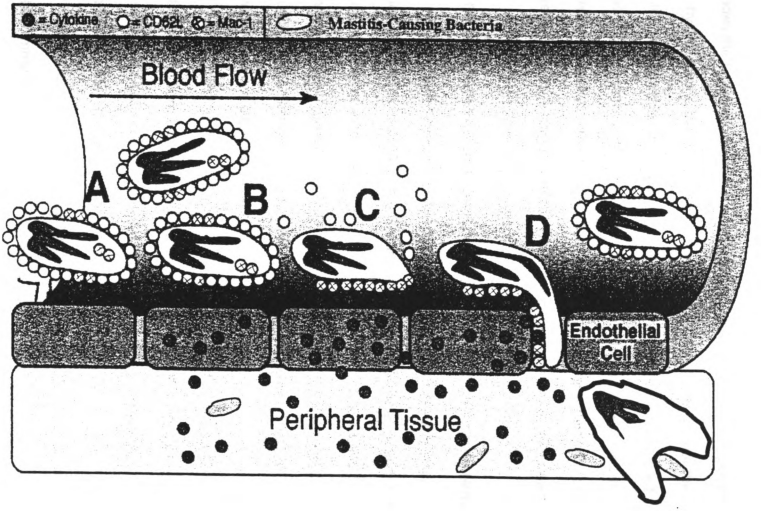
transmembrane molecules. When CD18 pairs with CD11a it is called leukocyte function antigen 1 (LFA-1) and is expressed primarily by lymphocytes. A CD11b/CD18 heterodimer makes up the Mac-1adhesion complex which is expressed mainly by neutrophils; CD18 pairing with CD11c makes up the adhesion complex known as p150,95, and is expressed mainly by monocytes and macrophages (Gilbert et al., 1992; Gahmberg et al., 1998).

The importance of these integrins in immunocompetence is demonstrated in individuals with leukocyte adhesion deficiency (LAD in humans; bovine LAD or BLAD in cattle). This is an autosomal recessive disease resulting in abnormal folding of the β chain (CD18), and therefore abnormal (0) expression of the β2 integrin. Neutrophils and monocytes of affected individuals cannot migrate from the blood vessels into the tissues. LAD and BLAD individuals usually die at an early age from widespread opportunistic bacterial infections (Anderson et al., 1985; Schuster et al., 1992).

After strong adhesion of rolling (marginating) leukocytes to the activated endothelia, the cells migrate into the tissue along a chemotactic gradient of cytokines and chemokines (Figure 1.4D). Many of the chemokines that act as trigger factors also serve as chemotactic factors (Adams and Shaw, 1994). Without adequate expression of the adhesion molecule CD62L to slow circulating leukocytes, and CD18/CD11b to tightly adhere these leukocytes to the endothelium, these cells cannot enter the mammary gland where they are needed to ingest and kill mastitis-causing bacteria. The function of these

molecules makes them especially important indicators of innate immunocompetence.

Figure 1.4. Model of neutrophil migration through the vascular endothelium, involving regulation of the leukocyte adhesion molecules L-selectin (CD62L) and Mac-1 (CD11b/CD18). Circulating neutrophils expressing high amounts of CD62L tether to the vascular endothelium and roll along the surface under the shear force of blood flow (A). When these marginating cells come into contact with trigger factors (e.g. platelet-activating factor) from infection or inflammation (B) they begin to shed CD62L and up-regulate CD11b/CD18 in order to tightly adhere to the endothelium (C). The neutrophil is then able to migrate through the endothelium and into the tissue where it can begin to ingest and kill bacteria.


= Cytokine
= CD62L
= Mac-1
Mastitis-Causing Bacteria
Blood Flow
A
B
C
D
Endothelial Cell
Peripheral Tissue

C. Immunocompetency of the periparturient dairy cow

As described above, migration of neutrophils into mammary tissue provides the first immunological line of defense against bacteria that penetrate the physical barrier of the teat canal. If neutrophils are unable to clear the infection, the adaptive arm of the immune system, which has encountered antigen in the lymph system, becomes activated to help fight the infection. However, around the time of parturition, there are many dysfunctions in both innate and adaptive immunity that prevent a successful immune response (Table 1.2).

The link between the GH/IGF-I axis and immunocompetence

GH and IGF-I have immunoenhancing effects in humans and many species of domestic animals. Both GH and IGF-I influence acquired and innate immune responses, local inflammatory responses, tissue repair, wound healing, and hematopoeisis (Burton et al., 1994). Therefore, it seems logical that uncoupling of the GH/IGF-I axis by NEB could impair immunocompetence.

A. Evidence from GH-deficient animals

GH-deficient animals such as hypophysectomized rodents, Snell-Bagg dwarf mice, and some breeds of dogs have well characterized immuno-deficiencies. These include underdeveloped bone marrow (especially mononuclear cells), reduced weight and cellularity of the thymus, spleen, and lymph nodes, decreased blood lymphocytes and neutrophils, deficient cell-mediated immune responses, and decreased T-cell dependent antibody responses (reviewed by Burton et al., 1994). Of particular importance is that in

most cases, treatment with exogenous GH and (or) IGF-I reconstitutes the immune systems of these animals, suggesting that these hormones are vital for normal immune function.

In addition, treatment of hypophysectomized dogs and mice with GH or IGF-1 restores thymic and spleen size (Kelley, 1989; Arkins, 1993). Rats given antisera to GH develop thymic atrophy and wasting disease (Arkins, 1993). Thymic involution was reversed in aged rats implanted with GH-secreting cells (Kelley et al., 1990) and aged humans have deficient natural killer cell activity which can be augmented by exogenous GH (Kelley, 1990).

B. Evidence from lymphoid cell lines, humans and rodents

GH receptor expression has been studied in several cell lines. One such cell line is the transformed human lymphoid cell line called IM-9. Cultured IM-9 cells bind GH with high affinity and specificity (Lesniak et al., 1974), and these GH receptors are down-regulated by increasing the concentration of GH in the support medium (Lesniak and Roth, 1976). Radioimmunoassays demonstrate that cultured rat bone marrow cells, thymocytes, splenocytes, and peripheral blood cells secrete immunoreactive GH. Leukocyte derived GH augments DNA synthesis in rat splenocytes and this effect is abrogated in the presence of anti-rat GH antibody (Weigent and Blalock, 1991). Similar results occur in human peripheral blood mononuclear (PBMC) cells. Approximately 1% of unstimulated PBMC secrete GH and that GH secretion is increased at least 100% by stimulation with T-cell mitogens (Varma et al., 1993). Additionally, stimulation by PHA increases the number of GH-secreting cells by about 50% (Varma et al.,

1993). Secretion of GH also increases in PHA-stimulated human PBMC by addition of GH but not IGF-I (Varma et al., 1993).

The effects of GH on lymphoid cells seem to be mediated by paracrine synthesis of IGF-1 (Arkins et al., 1993; Geffner et al., 1990). In other words, some immune cells, in response to GH binding, are able to produce IGF-1 that acts on nearby cells. Expression of IGF-I has been described in different lymphoid organs and in several leukocyte subpopulations such as PBMC, thymic lymphocytes, splenic lymphocytes, monocytes, and macrophages. IGF-I mRNA is expressed strongly in activated macrophages but weakly in lymphocytes (Kooijman et al., 1996). Several studies indicate that TNF-α is a key cytokine in the regulation of IGF-I production by macrophages (Noble et al., 1993; Fournier et al., 1995). IGF-I transcripts exist in the spleen and thymus; expression of IGF-I in these organs is decreased by hypophysectomy and restored with exogenous GH (Murphy et al., 1987).

There is also evidence that GH and IGF-I affect the functional capacity of immune cells. Both GH and IGF-1 promote development of human myeloid cells *in vitro* (Merchav et al., 1988). Myeloid cells are the stem cells that give rise to monocytes and neutrophils, the primary responders to bacterial infections such as mastitis. GH and IGF-1 also stimulate monocyte migration (Wiedermann et al., 1993) and activate neutrophils and macrophages to produce superoxide anions and cytokines needed to kill invading bacteria and activate other effector and accessory cells that contribute to the inflammatory response (Saito et al., 1996). GH also increases antibody synthesis by B cells (Saito et al., 1996).

Wiedermann et al. (1993) demonstrated that GH is a potent chemoattractant for human monocytes *in vitro* and that *in vivo* a single dose of recombinant GH increased random migration of circulating monocytes. Not only has the importance of these hormones to immunity been demonstrated in laboratory animals and *in vitro*, but they also affect the bovine immune system.

C. Evidence from cattle treated with exogenous GH (bST)

When bovine GH was added to the culture medium of rat thymocytes poised at the S-phase of the cell cycle, it caused an increase in DNA synthesis, degree of mitosis, and division of the cells. Specific binding of bovine GH to bovine and murine thymocytes in culture also was observed when GH was added at physiological concentrations (Arrenbrecht, 1974). Blood polymorphonuclear leukocytes (PMN) collected from healthy cows 1 week after daily treatments with bST generated greater amounts of reactive oxygen species upon stimulation than prior to treatment with bST (Heyneman et al; 1989). Production of reactive oxygen species from milk PMN from experimentally induced mastitic cows also was greater for cows treated with bST than untreated controls (Burvenich et al., 1989).

In one study, lactating dairy cows were treated with 10.3 and 20.6 mg of bST daily for 266 consecutive days beginning between weeks 4 and 5 of lactation. The results showed that cultured blood mononuclear cells stimulated with ConA , but not unstimulated cells, had a 30% increase in blastogenesis relative to control cows (Burton et al., 1991). In the same study, the 20.6 mg d^{-1} dose caused a significant reduction in the fraction of lymphocytes and an

increase in neutrophils in the peripheral blood (Burton et al., 1992). It was suggested that for GH and IGF-I to act in such a profound manner on the immune system, these hormones must be able to bind to specific receptors.

D. Evidence that immune cells produce and(or) possess receptors for GH and IGF-I

The IM-9 cell line is used extensively for GH receptor studies. These cells express specific, high affinity receptors for GH with an estimated 4000 binding sites per cell (Lesniak, 1974). Binding sites for bovine GH are found on bovine and murine thymocytes (Arrenbrecht, 1974). In human PBMC, there is an average of 6800 binding sites for human GH per cell. Binding sites for human GH also exist on rat liver macrophages and on a murine thymic epithelial cell line (Kiess and Butenandt, 1985). Expression of GH receptors is higher in B cells than in T or natural killer cells (Valerio et al., 1997).

Receptors for IGF-1 are expressed on all major subpopulations of human PBMC (Stuart et al., 1991). In addition, human thymocytes at all developmental stages express IGF-I receptors. Expression of IGF-I receptors by human T-cells increases after stimulation with PHA (Kozak et al., 1987). The importance of IGF-I receptors in human peripheral blood T-lymphocyte proliferation is demonstrated by the finding that T lymphocytes cannot enter the S-phase when the expression of IGF-I receptors is blocked by anti-sense RNA (Reiss et al., 1992). Whereas only small numbers of receptors exist on resting T-lymphocytes, these increased significantly after mitogen stimulation. Receptors for IGF-I also are found in B-lymphocytes and monocytes with an estimated 1250 binding sites per cell when expressed in total mononuclear cells or 4940 binding sites per monocyte (Stuart

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et al., 1991). Elevated local concentrations of IGF-I down-regulate IGF-I receptors on circulating human mononuclear cells both *in vitro* and *in vivo* and exert similar effects on mitogen-activated T-lymphocytes *in vitro*. Zhao et al. (1992) determined that there are high-affinity, IGF-I specific receptors on bovine PBMC and that binding of receptor and ligand increases uptake of tritiated thymidine by the cells.

In summary, IM-9 cells, untransformed lymphocytes from peripheral blood, splenocytes, polymorphonuclear and mononuclear leukocytes, and tissue macrophages from several species, in different physiological states, bind and respond to GH and IGF-I. The receptor hormone binding characteristics in these cells appear to be similar to those described for non-lymphoid cells. Immune cells also produce and secrete both GH and IGF-I and these hormones have the ability to mediate immune cell function. Studies also have shown acute down-regulation of receptors for GH and IGF-I with increasing local concentrations of these hormones. This suggests the possibility that local and (or) circulating GH and IGF-I may regulate immune function. This may be of particular importance in times of nutritional stress such as NEB in cattle.

Hypothesis and objectives of the current study

The overall hypothesis of this thesis is that NEB alters the expression of GH and (or) IGF-I receptors on bovine blood leukocytes (Figure 2.1; Chapter 2). The secondary hypothesis is that NEB also alters gene expression of key adhesion (CD62L, CD18, CD11b) and antigen presenting molecules (MHC class I, MHC class II) by bovine blood leukocytes. The key implication of these

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hypotheses is that NEB in periparturient dairy cows contributes to infectious disease susceptibility via uncoupling of the GH/IGF-I axis in the immune system.

To test this hypothesis, specific objectives were designed.

Objective 1: To develop a model of periparturient negative energy balance that would both uncouple the GH/IGF-I axis and eliminate possible confounding effects of reproductive and other metabolic hormones.

Objective 2: To develop immunostaining and fluorescence-activated flow cytometric protocols to monitor hormone receptors, adhesion molecules and antigen-presenting molecules on bovine blood leukocytes.

Objective 3: To determine effects of NEB on bovine leukocyte expression of GH and IGF-I receptors.

Objective 4: To determine effects of NEB on bovine leukocyte expression of adhesion molecules and antigen presenting molecules.

Results of these studies could implicate NEB as a major contributor to infectious disease susceptibility in dairy cattle. The importance of these findings could emphasize the need for improved management to minimize the severity and number of animals undergoing NEB. This also could have implications for malnutrition in other species such as humans.

Chapter 2

EFFECT OF NEGATIVE ENERGY BALANCE ON THE EXPRESSION OF HORMONE RECEPTORS BY BOVINE BLOOD LEUKOCYTES

Abstract

Sixteen yearling Holstein steers were fed for 210% or 60% of maintenance requirements in a crossover design with two treatment periods of 3 weeks, separated by a 2-week adjustment period, to impose positive (PEB) or negative energy balance (NEB), respectively. Blood was collected and analyzed for serum concentrations of nonesterified fatty acids (NEFA), growth hormone (GH), and insulin-like growth factor-I (IGF-I). Leukocytes were isolated and analyzed for expression of receptors for GH and IGF-I using immunostaining and flow cytometric analysis. Compared with PEB, NEB decreased the concentration of IGF-I and increased the concentration of NEFA in serum ($P < 0.05$). A smaller percentage of circulating blood lymphocytes expressed GH receptor ($P = 0.002$) during NEB than during PEB. However, in those lymphocytes that expressed GH receptor, expression tended to be greater higher during NEB than during PEB ($P = 0.087$). Compared with PEB, NEB also decreased the percentage of blood monocytes expressing IGF-I receptor ($P = 0.009$). The results of this study indicate that NEB affected expression of GH and IGF-I receptors by some mononuclear leukocytes in cattle, but that it has no effect on expression of these hormone receptors in neutrophils.

Introduction

Nutrition plays a key role in the effectiveness of the immune response, and malnutrition is the most common cause of immunodeficiency worldwide (Chandra, 1997). Children suffering from protein-energy malnutrition (PEM) have increased susceptibility to and severity of infectious disease. The ability of neutrophils and monocytes of these children to kill bacteria is impaired. They also have a lower blood lymphocyte count and a decreased antibody response to tetanus toxin compared with healthy children (Chandra, 1973).

A similar condition of PEM is observed in dairy cows around the time of parturition. At this time, dairy cows are usually in negative energy and protein balance. This is due to decreased dry matter intake and an increase in the demand for energy and protein at the onset of lactation (Grummer, 1995). The concentration of circulating NEFA is elevated from the mobilization of body reserves to meet the increased energy demand. Dairy cows also are immunosuppressed and therefore more susceptible to bacterial infection around the time of parturition (Lee and Kehrli, 1998; Detilleux et al., 1995). On commercial farms, cows with high serum NEFA before calving have an increased incidence of mastitis and retained placenta after calving (Dyk et al., 1995). This relationship is consistent with the idea that NEB is partly responsible for the immunosuppression of dairy cows around calving.

One possible mechanism by which NEB might affect immune function is via metabolic hormones such as GH and IGF-I. Blood and tissue concentrations of IGF-I are regulated in part by blood GH concentration. Exogenous GH or GH

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secreted from the pituitary binds to specific receptors in the liver causing synthesis of IGF-I and increased circulating concentrations of IGF-I (Cohick et al., 1989; McGuire et al., 1992a). However, NEB can uncouple the GH/IGF-I axis in cattle. Circulating concentration of IGF-I decreases significantly during NEB even though circulating GH may be high (Yung et al., 1996; McGuire et al., 1992a,b; Clemmons and Underwood, 1991; VandeHaar et al., 1995; Sharma et al., 1994). Uncoupling of the GH/IGF-I axis during NEB is partly due to down-regulation of GH receptors in the liver (Yung et al., 1996; McCusker, 1998).

Cultured human lymphoid cells, untransformed lymphocytes from peripheral blood, splenocytes, polymorphonuclear and mononuclear leukocytes, and tissue macrophages from several species, including cattle, in different physiological states bind and respond to GH and IGF-I via specific receptors (Lesniak and Roth, 1976; Lesniak et al., 1974; Stuart et al., 1991; Zhao et al., 1992). Immune cells also produce and secrete both GH and IGF-I, and these hormones have the ability to mediate immune cell function. Studies also have shown acute down-regulation of receptors for GH and IGF-I with increasing local concentrations of these hormones (Lesniak and Roth, 1976; Lesniak et al., 1974; Stuart et al., 1991; Zhao et al., 1992). This suggests the possibility that local and (or) circulating GH and IGF-I may regulate immune function. If NEB uncouples the GH/IGF-I axis at the liver via down-regulation of receptors, perhaps the same is true in the immune system. Decreased GH or IGF-I binding would prevent the immunoenhancing effects of these hormones and could result in decreased immunocompetence and increased susceptibility to disease.

The hypothesis of this study was that NEB decreases expression of GH and IGF-I receptors by bovine blood leukocytes (Figure 2.1). The objective was to determine if NEB alters the expression of GH and IGF-I receptors by bovine blood leukocytes.

Materials and Methods

A. Animals and Diets

Yearling Holstein steers (n=16) weighing approximately 365 kg were used as the experimental animal model of NEB. At the start of the trial, steers were randomly assigned to two groups and fed either a PEB diet (210% of maintenance requirements) or a NEB diet (60% of maintenance requirements; Table 2.1). Both diets met or exceeded NRC requirements for vitamins and mineral elements (NRC, 1989), and both diets had similar protein-energy ratios. The steers were fed diets in a crossover design with two treatment periods of 3 weeks separated by a 2-week adjustment period (Figure 2.2). Diets were fed as a complete mix in two meals per day at 0800 and 1700 h. The All University Committee on Animal Use and Care of Michigan State University approved animal care and dietary treatments, as well as the blood sampling protocol. Steers were fed and housed individually at Michigan State University's Beef Cattle Research Center. Individual intakes were measured daily and body weight (BW) was recorded two consecutive days per week. Maintenance requirement was calculated using the following equation (NRC, 1989):

$$NE_m = (.086)BW^{.75}.$$

Energy balance for the steers fed for PEB was calculated as follows:

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NE_m (Mcal/kg) of the diet / NE_m requirement = feed for maintenance

(Feed intake – Feed for maintenance)*NE_g of the diet (Mcal/kg) = Energy balance.

Energy balance for the steers fed for NEB was calculated by simply subtracting the amount of NE_m required from the amount of NE_m consumed in the diet.

B. Blood Sampling

Blood was collected on day -7, -4, 0, 4, 7, 14, and 21 relative to each of the two treatment periods. At 0700 h on sample days (prior to feeding), blood (6 ml) was collected by jugular venipuncture into Vacutainer® blood collection tubes containing acid-citrate-dextrose anticoagulant. This blood was used for blood leukocyte counts and immunostaining of GH and IGF-1 receptor on leukocytes. An additional 10 ml of blood were collected into Vacutainer® blood collection tubes containing no anticoagulant and stored overnight at 4°C; serum was harvested by centrifugation for 25 min at 1550 x *g* and stored at -20°C. Serum was analyzed for NEFA, GH, and IGF-1 concentrations. Additionally, on the last day of the second treatment period, 10 ml of blood were collected at 1200 h (3 h post-feeding) for determination of serum GH.

C. Hormone and NEFA Assays

The concentration of NEFA in serum was determined enzymatically (NEFA-C Kit; Wako Pure Chemicals, Inc., Osaka, Japan, adapted for use in microtiter plates; Johnson and Peters, 1993). Serum was assayed for IGF-I concentrations by first removing binding proteins using acid/ethanol extraction (Bruce et al., 1991) and using the resulting extracts in a radioimmunoassay.

Radioimmunoassay was performed as in GroPep Pty Ltd (Growth Factors Products and Protocols, Adelaide, Australia) with GroPep's IGF-I standard and primary antibody, but modified as in Sharma et al. (1994) with *Staphylococcus aureus* used in place of the secondary antibody. Serum GH was determined by radioimmunoassay as in Gaynor et al. (1995).

D. Leukocyte Counting and Standardization of Cell Numbers

Leukocytes were isolated from whole blood of each steer at each sampling time. Briefly, 5 ml of acid citrate dextrose-anticoagulated blood per steer was added to a 50 ml conical tube. Red blood cells were lysed for 90 s by adding 10 ml of cold hypotonic lysing solution (10.6 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4, pH 7.3). Isotonicity was restored by adding 20 ml of cold hypertonic restoring solution (10.6 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4, 0.43 *M* NaCl, pH 7.3). Cellular debris was removed by centrifugation at 350 x *g* for 5 min at 22°C and leukocytes were resuspended in 2 ml of phosphate buffered saline (pH 7.5) that contained 0.1% bovine serum albumin (Sigma, St. Louis, MO; wash solution) and transferred to a 12x75 polystyrene tube (Fisher Scientific, Pittsburgh, PA). Tubes were centrifuged again, supernatant was aspirated and cells suspended in 1 ml of wash solution. Total leukocytes were counted using a hemacytometer (Hausser Scientific Co., Horsham, PA), and 5 x 10^5 cells were added per well to a 96-well round-bottom microtiter plate (Nalgene Nunc International, Milwaukee, WI) for fluorescent staining.

E. GH and IGF-I Receptor Immunostaining Procedure

Prepared leukocytes (above) were centrifuged at 350 x *g* for 5 min at 22°C, supernatant was aspirated and primary monoclonal antibodies were added. The antibodies used were anti-IGF-I receptor IgG1 (clone 1H7 from Pharmingen, San Diego, CA), and anti-GH receptor IgG2a (clone R1-M1-B11, donated by Dr. Wesley Warren, Monsanto Co., St Louis, MO). One well per steer received 100 μl of a 1:400 dilution (in wash solution) of anti-IGF-I receptor, and one well per steer received 100 μl of a 1:10 dilution (in wash solution) of anti-GH-receptor. Plates were incubated in the dark at 4°C for 30 min and then centrifuged at 350 x *g* for 5 min at 22°C. Supernatant was aspirated and cells were washed with 200 μl of wash solution. Secondary antibodies were then added. All secondary antibodies were conjugated to fluorescein isothiocyanate (FITC; Figure 2.3). Secondary antibodies (100 μl) were added to the appropriate wells. The secondary antibody for IGF-I receptor staining was a 1:20 dilution (in wash solution) of anti-mouse IgG1-FITC (clone M32004 from Caltag, San Francisco, CA). The secondary antibody for GH receptor staining was a 1:10 dilution (in wash solution) of anti-rat IgG2a-FITC (clone RG7 from Pharmingen, San Diego, CA) used at 10 μl/well. A 7μl/ml dilution (in wash solution) of irrelevant mouse IgG1 FITC Isotype Control (0.35 μl/well ; clone X0927 from Dako, Carpinteria, CA) was added to one well per steer and served as the negative control. Plates were incubated in the dark at 4°C for 15 min and then centrifuged at 350 x *g* for 5 min. Supernatant was aspirated and cells were fixed with a 1:10 dilution (in H_2O) of FACS Lysis solution (15 μl/well, Becton Dickinson,

San Jose, CA) for 10 min in the dark at 22°C to stop the metabolic functions of the cells. Plates were centrifuged again, supernatant was aspirated, cells were washed one final time in 200 μl wash solution, and cells were then resuspended in 200 μl of sheath fluid (Isoton II, Coulter Diagnostics, Hialeah, FL) for immediate flow cytometric analysis.

F. Flow cytometric analysis of GH and IGF-I receptors

The FACSCalibur fluorescence activated flow cytometer and the acquisition package of CellQuest software (Becton Dickinson, San Jose, CA) were used to acquire and analyze the leukocyte GH and IGF-I receptor data. Unstained cells in two wells per steer were used to check the forward scatter (FSC = size characteristics) and side scatter (SSC = granularity characteristics) parameters of the leukocytes. These parameters allowed individual animal variation in physical leukocyte characteristics to be adjusted in FSC-SSC density dot plots (Figure 2.4). Detectors for FITC fluorescence were adjusted in preliminary experiments for optimal demonstration of green fluorescence. Detector settings were not altered during the experiment. Data from 5000 events (approximately 5000 cells) per well were acquired. Neutrophils, monocytes, and lymphocytes were gated out based on well-known forward (size) and side scatter (granularity) characteristics on density dot plots (Figure 2.4; Burton and Kehrli, 1995). The FITC fluorescence histograms of cells in each gated region were then plotted (Figure 2.5) and geometric mean fluorescence intensities (MFI) were computed from the Y-axis (cell counts) and the X-axis (intensity channel). The region of background fluorescence based on the negative control was identified

(solid green peak in Figure 2.5) and events to the right of a marker (M1) were considered positive fluorescence events (Figure 2.5). The percentage of gated cells that fell within this positive region was determined along with their MFI. The position of M1 was not altered throughout the duration of the trial so that any shift to the left or right in positive fluorescence intensity over time (treatment effect) was detectable (indicating down or up-regulation of the hormone receptors respectively).

G. Leukocyte Counts

Neutrophil, monocyte, and lymphocyte counts (number of cells/ml) were determined by multiplying total leukocyte counts (number of cells/ml) by the percentage (%) of each cell type determined using physical characteristics (FSC and SSC) of unstained cells (Figure 2.4).

H. Statistical Analyses

Specific questions were asked to aid in the development of appropriate statistical models. Questions are as follows:

1) is there a mean difference between NEB and PEB in treatment period 1;
2) is there a mean difference between NEB and PEB in treatment period 2;
3) is there a mean carryover effect from treatment period 1 into treatment period 2; and,
4) is there an overall mean difference between PEB and NEB?

Statistical Analysis Software (SAS® PROC MIXED) was used to analyze data by repeated measures analysis correcting for correlated error within steer (SAS®, 1996). The linear model included fixed effects of group, day (relative to the start of the trial), and plate (flow cytometric data were acquired using two 96-well microtiter plates), the interactions of group by day, plate by day, and plate by group (Model 1). Repeated measures within animal were modeled as a heterogeneous compound symmetry structure such that the random animal effect was fitted indirectly and an allowance was made for unequal residual variances across days (Littell et al., 1998). The test term (error) for group, plate, and group by plate was animal within group by plate. Any model term involving day was tested against the residual variance. Treatment was indirectly fitted through group and group by day model terms. The model (Model 2) for leukocyte counts, IGF-I, GH, energy balance, body weight, and NEFA did not include plate or interactions involving plate. These data, along with MFI data were log transformed because of erratic heterogeneous variance observed upon initial examination of empirical distribution of the residuals. All percentage data were converted to decimal values and transformed as the inverse sine of the square root.

Contrasts were derived to provide estimates for the treatment effects in order to address the questions described previously. This was done by using the following equations in which $\mu_{i,j}$ represents the LSM of the *i*th group on the *j*th day of the study. For example $\mu_{A,28}$ represents the least squares mean (LSM) of group A on day 28 relative to the start of the trial (refer to Figure 2.2).

In order to address the first two questions, the average PEB and NEB values of each dependent variable for each treatment period were estimated. These values were then contrasted as follows:

$\frac{1}{2}$ ($\mu_{A,28} + \mu_{A,35}$) – $\frac{1}{2}$ ($\mu_{B,28} + \mu_{B,35}$) = estimated difference between NEB (group A) and PEB (group B) in treatment period 1; and,

$\frac{1}{2}$ ($\mu_{A,63} + \mu_{A,70}$) – $\frac{1}{2}$ ($\mu_{B,63} + \mu_{B,70}$) = estimated difference between PEB (group A) and NEB (group B) in treatment period 2.

To determine if carryover effects were present, thereby answering question three, similar procedures were followed for data obtained during the adjustment period when both groups were in PEB. Individual estimates of the difference in the dependent variable of the two groups were calculated. Estimates were determined for day 39, 43, and 46 using the following contrasts:

$\mu_{A,39} - \mu_{B,39}$ = estimated difference between group A and group B on day 39;

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 43; and,

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 46.

The following contrast also was developed to compare the LSM for each group across the 3 sample days during the adjustment period:

1/3 ($\mu_{A,39} + \mu_{A,43} + \mu_{A,46}$) – 1/3 ($\mu_{B,39} + \mu_{B,43} + \mu_{B,46}$) = estimated difference between group A and group B across days 39, 43 and 46.

The results of these contrasts showed that some of the dependent variables measured (GH receptor, CD18, CD11b, MHC class I, and plasma GH pre-feeding) were different between the two groups on day 46. In order to simplify analyses, all data were corrected for this carryover when estimating the overall difference between PEB and NEB.

To correct for carryover effects and therefore answer question four, the LSM for the last two samples of each treatment period (day 28, 35 and 63, 70) for each group (A or B) were averaged as follows:

A: ½ ($\mu_{A,28} + \mu_{A,35}$) = estimated LSM for group A, treatment period 1(NEB);

B: ½ ($\mu_{A,63} + \mu_{A,70}$) = estimated LSM for group A, treatment period 2(PEB);

C: ½ ($\mu_{B,28} + \mu_{B,35}$) = estimated LSM for group B, treatment period 1 (PEB); and,

D: ½ ($\mu_{B,63} + \mu_{B,70}$) = estimated LSM for group B, treatment period 2 (NEB).

This resulted in two treatment means per group, one for each treatment period or one PEB mean and one NEB mean. Similarly, the average of the LSM from the two samples prior to the start of the each treatment period (day 10, 14 and 46, 50) for each group were calculated:

E: ½ ($\mu_{A,10} + \mu_{A,14}$) = estimated LSM for group A, pre-treatment period 1 (pre-NEB);

F: ½ ($\mu_{A,46} + \mu_{A,50}$) = estimated LSM for group A, pre-treatment period 2 (pre-PEB);

G: ½ ($\mu_{B,10} + \mu_{B,14}$) = estimated LSM for group B, pre-treatment period 1 (pre-PEB); and,

Tha

trea

Data

were

H: ½ ($\mu_{B,46}$ + $\mu_{B,50}$) = estimated LSM for group B, pre-treatment period 2 (pre-NEB).

These averages were combined in a contrast that corrected the LSM of the treatment periods for pre-treatment values:

½ (A - E) + ½ (D - H) = estimate of overall NEB adjusted for pre-treatment means;

½ (C - G) + ½ (B - F) = estimate of overall PEB adjusted for pre-treatment means; and,

[½ (A - E) + ½ (D - H)] – [½ (C - G) + ½ (B - F)] = estimated overall difference between NEB and PEB adjusted for pre-treatment means.

Data are presented as LSM with 95% upper and lower confidence intervals and were backtransformed to be presented in original units.

Model 1

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \alpha\beta_{ij} + \alpha\gamma_{ik} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *k*th plate and the *l*th steer. The dependent variables monitored included:

percentage of monocytes, lymphocytes and neutrophils expressing IGF-I and GH receptor; and,

mean fluorescence intensity of IGF-I and GH receptor staining in monocytes, lymphocytes and neutrophils.

μ = overall mean

α_i = fixed effect of group (*I* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

γ_k = fixed effect of plate (*k* = 1,2)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

$\alpha\gamma_{ik}$= interaction of *i*th group and *k*th plate

$\beta\gamma_{jk}$= interaction of *j*th day and *k*th plate

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all *i,j,k,l* and equally correlated across time within the *l*th steer, allowing for heterogeneity in variance over time (i.e., the repeated measures option of SAS PROC MIXED, Type=CSH).

Model 2

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *l*th steer. The dependent variables monitored included:
serum NEFA;
body weight;
calculated energy balance;
serum IGF-I;
serum GH;
total circulating leukocytes;
circulating lymphocytes;
circulating monocytes; and,
circulating neutrophils.

μ = overall mean

α_i = fixed effect of group (*i* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all i,*j*,*k*,*l* and equally correlated across time within the *l*th steer, allowing for heterogeneity in variance over time (i.e. the repeated measures option of SAS PROC MIXED, Type=CSH).

Results

A. Energy Balance

Steers fed the PEB diet gained 1.5 ± 0.3 kg per day and steers fed the NEB diet lost 1.2 ± 0.2 kg per day. Steers fed the NEB diet had greater (*P* = 0.0001) circulating concentrations of NEFA (800 ± 1.1 μEq/L) than steers fed the PEB diet (150 ± 1.2 μEq/L). Calculated energy balance was 5.7 ± 0.3 Mcal/day

for the steers fed the PEB diet and –3.2 ± 0.3 Mcal/day for those fed the NEB diet (P = .0001; Figure 2.5).

B. Serum Hormones

Negative energy balance caused by the NEB diet decreased the circulating concentration of IGF-I in both treatment periods ($P < 0.05$; Figure 2.7). The LSM of IGF-1 during NEB was 212 ± 12 pg/ml, and during PEB was 297 ± 12 pg/ml demonstrating uncoupling of the GH/IGF-I axis. The LSM of the circulating concentration of GH prior to feeding (0700 h) was 2.5 ± 1.2 ng/ml during NEB which tended to be slightly lower ($P = 0.06$; Figure 2.6) than the concentration during PEB (3.3 ± 1.2 ng/ml). However, the samples taken at 1200 h on the last day of the second treatment period (3 h post-feeding) indicated that the circulating concentration of GH was greater (6.9 ± 1.0 ng/ml) in the NEB steers than the PEB steers (2.7 ± 0.5 ng/ml; $P = 0.005$).

C. Leukocyte Counts

Analysis of total leukocyte counts showed that NEB increased the number of circulating lymphocytes by approximately 17% ($P = 0.007$), but did not affect the numbers of neutrophils or monocytes.

D. IGF-I Receptor Expression

Energy balance did not alter the MFI of IGF-I receptor expression on neutrophils, monocytes and lymphocytes. Also, NEB did not change the percentage of neutrophils or lymphocytes expressing IGF-I receptor. However, NEB slightly decreased the percentage of monocytes expressing IGF-I receptor (Table 2.2; P =0.009).

E. GH Receptor Expression

Negative energy balance did not alter the MFI of GH receptor expression on neutrophils and monocytes. However, NEB tended to increase MFI of GH receptor expression by lymphocytes (Table 2.3; $P = 0.06$). Negative energy balance did not alter the percentage of neutrophils or monocytes expressing GH receptor. Negative energy balance decreased the percentage of lymphocytes expressing GH receptor (Table 2.3; $P = 0.002$). This decrease was observed in the first treatment period at the first sample (d 4) and was maintained throughout the first treatment period and even after the steers were switched to the PEB diet. The pretreatment expression level was not reached until d 7 of the second treatment period (Figure 2.8).

Discussion

NEB in sheep and cows has increased serum GH, decreased GH receptor mRNA and GH binding in the liver and, in turn, decreased hepatic IGF-I mRNA and serum IGF-I (Yung et al., 1996; McGuire et al., 1992a,b; VandeHaar et al, 1995; Pell et al., 1993; Elsasser et al., 1989; Bass et al., 1990; Breier et al., 1989; Clemmons and Underwood, 1991). In the present study NEB decreased the percentage of bovine blood lymphocytes expressing GH receptors and slightly decreased the percentage of bovine blood monocytes expressing IGF-I receptors.

In this study, NEB decreased circulating IGF-I, increased serum NEFA and caused a loss of 1.2 kg/day in body weight. Compared with PEB, the serum concentration of GH during NEB was slightly lower before feeding and higher 3 h

post-feeding. The pulsatile nature of GH secretion (Davis et al., 1984) makes interpretation of these GH data difficult because GH was measured as a single blood sample per day. To accurately assess GH concentration, blood samples should be collected several times over a 6 to 12 h time period (Davis et al., 1984). A previous study using a similar model of NEB in cattle showed that NEB increased the mean serum GH concentration over a 10-h period (VandeHaar et al., 1995). Thus, my results suggest that the GH/IGF-I axis was uncoupled in the liver during NEB most likely because of decreased GH receptor expression and (or) post-receptor lesions (Yung et al., 1996; McGuire et al., 1992a,b; VandeHaar et al, 1995; Pell et al., 1993; Elsasser et al., 1989; Bass et al., 1990; Breier, et al., 1989; Clemmons and Underwood, 1991).

Changes in circulating concentrations of GH and IGF-I due to NEB may affect leukocytes and thus contribute to susceptibility to infectious diseases such as mastitis. In this study, NEB decreased the percentage of circulating lymphocytes expressing GH receptor from 50 to 42% (Table 2.3). There are two possible explanations for this decrease. A specific subpopulation of lymphocytes may have down-regulated GH receptor in response to NEB or, perhaps a subpopulation of lymphocytes left the circulation in response to NEB. There a many subpopulations of lymphocytes and it is feasible that a population down-regulated the GH receptor due to internalization of the bound hormone (Allevato et al., 1995). Addition of GH to cultured human lymphoid IM-9 cells causes down-regulation of the GH receptor (Lesniak and Roth, 1976). Once GH binds to the GH receptor, it is internalized, suggesting that the duration and degree of

sensitivity to GH may be regulated at the cellular level (Lesniak and Roth, 1976). A study using bulls found that B-lymphocytes made up about 10% of the circulating population of leukocytes (Burton and Kehrli, 1995). Because approximately 10% fewer circulating lymphocytes expressed GH receptor, B-lymphocytes could be the affected population.

It is also possible that the decreased proportion of lymphocytes expressing GH receptor during NEB may be the result of a population of lymphocytes leaving the peripheral blood. This is consistent with a study in which exogenous GH reduced the number of lymphocytes in the peripheral blood of cows (Burton et al., 1992). In the present study, a loss of lymphocytes from the total population that exhibited low intensity staining (i.e., less GH receptor per cell) might account for the small increase in MFI of the remaining cells that expressed GH receptor. However, this scenario is unlikely because NEB increased the number of circulating lymphocytes by 17%.

Although serum NEFA, average daily gain, and circulating concentration of IGF-I returned to PEB values shortly after refeeding, it is noteworthy that the percentage of lymphocytes expressing the GH receptor remained lower than that of the PEB group well after the end of the first treatment period (Figure 2.8). GH receptor expression did not return to pretreatment abundance until the second treatment period had begun. This suggests that the affected immune cells take longer to recover from NEB than other physiological indicators and that two weeks was not long enough for adequate recovery of the immune system before initiating the second treatment period. If a population of lymphocytes did leave

the peripheral blood, the delay in recovery following termination of NEB may have been due to a lag time needed for thymic release of new lymphocytes and (Morrison, 1986).

The observed decrease in serum IGF-I may have caused some immune cells to decrease their normal metabolic activity. Circulating IGF-I normally enhances both cell proliferation and differentiation via the type I IGF receptor (Bjerknes and Aarskog, 1995; Clark et al., 1993; Scheven and Hamilton, 1991; Merchav et al., 1988; McCusker, 1998). When immune cells terminally differentiate or become quiescent, it results in decreased number or loss of the type I IGF receptor (McCusker, 1998). The slight decrease in the percentage of monocytes expressing IGF-I receptor during NEB may be the result of monocytes becoming quiescent from decreased circulating IGF-I. This may be a homeorhetic response that would allow preferential use of serum IGF-I during NEB for maintenance of the animal. However, it is more likely that this small of a decrease might have been due to random chance.

NEB affected the percentage of mononuclear cells expressing both the IGF-I and GH receptors, but there appeared to be no effect of GH/IGF-I uncoupling on expression of these receptors by neutrophils. Therefore, it seems unlikely that these changes would have a large impact on innate immune functions. However, these changes in combination with other hormonal factors involved in malnutrition, such as cortisol, may cause generalized immunosuppression. Furthermore, the steers in this study were not given any experimental immune challenge nor did they exhibit any symptoms of observable

(ie., clinical) disease. Previous studies have focused mainly on the ability of GH and IGF-I to impact activated leukocytes or leukocytes that have encountered a pathogen (Chapter 1). It is possible that although only small changes in leukocyte receptor expression were observed in these healthy animals, a larger response might be observed if these steers were presented with a significant (ie., clinically observable) immune challenge. Also, it is clear that the adjustment period between the two treatment periods should have been lengthened to ensure adequate recovery of the immune system. It may also be possible that 3 weeks was not long enough to affect expression of these receptors as seen in the circulation.

The use of steers in this study was intended to prevent the interaction of NEB with these endocrine changes with the exception of GH and IGF-I. However, in so doing, the right combination of endocrine changes needed for decreased immunocompetence may not have been included. Therefore, this study may need to be done in periparturient dairy cows.

Future studies should examine the impact of long term NEB on expression of these receptors by bovine blood leukocytes. In addition, regulation of both GH and IGF-I receptor during NEB in response to an immune challenge, as well as changes in expression of these receptors in specific subpopulations of mononuclear leukocytes should be examined.

Chapter Two

Tables and Figures

Table 2.1. Diet composition[1] and nutrient intake of the negative (NEB) and positive (PEB) energy balance diets.

	PEB	NEB
% of Maintenance	210	60
Diet Composition, % of DM		
Grass Hay, chopped	21	43
Corn Silage	31	42
Ground Corn	35	7
Soybean Meal (44% CP)	11	5
Vitamin and Mineral Mix[1]	1.8	2.0
ME, Mcal/kg	3.0	2.3
CP, % of DM	14	12
Nutrient Intake		
Feed Intake, kg DM/day	7.5	2.2
ME intake, Mcal/day	22.5	5.1
Vitamin A, IU/day	19650	13200
Vitamin D, IU/day	2850	1936
Vitamin E, IU/day	164	110
Lipid, kg DM/day	0.25	0.06
Ca, g/day	29	19
P, g/day	26	14
Mg, g/day	13	7
K, g/day	85	46
Na, g/day	14	9.9
Cl, g/day	29	20
S, g/day	11	4
Co, mg/day	0.75	0.51
Cu, mg/day	110	69
Fe, mg/day	1300	700
I, mg/day	4.4	3.0
Mn, mg/day	410	270
Se, mg/day	2.3	1.50
Zn, mg/day	360	240

[1]Calculated based on composition of diet ingredients.

Table 2.2. Least squares means of insulin-like growth factor I receptor (IGF-IR) expression, measured as percent of positively stained cells and mean fluorescence intensity (MFI), for bovine blood leukocytes from steers in positive (PEB) and negative energy balance (NEB). Also shown are P-values for the treatment effect.

	PEB (95% Confidence Limit)	***NEB*** (95% Confidence Limit)	***P-value***
% of lymphocytes expressing IGF-IR	24.7 (13.8, 37.8)	28.2 (15.9, 41.5)	0.6
% of monocytes expressing IGF-IR	89.5 (86.2, 92.8)	86.9 (83.3, 90.6)	0.009
% of neutrophils expressing IGF-IR	99.2 (98.5, 99.7)	99.3 (98.5, 99.7)	0.9
Lymphocyte MFI	10.5 (8.9, 34.5)	10.5 (9.0, 12.3)	0.7
Monocyte MFI	30.0 (26.1, 34.5)	30.3 (26.3, 34.8)	0.9
Neutrophil MFI	16.6 (14.0, 19.7)	18.4 (15.5, 21.5)	0.3

Table 2.3. Least squares means (±SEM) of growth hormone receptor (GHR) expression, measured as percent of positively stained cells and mean fluorescence intensity (MFI), for bovine blood leukocytes from steers in positive (PEB) and negative energy balance (NEB). Also shown are P-values for the treatment effect.

	PEB (95% Confidence Limit)	***NEB*** (95% Confidence Limit)	***P-value***
% of lymphocytes expressing GHR	39.4 (24.8, 55.1)	31.0 (16.6, 47.7)	.002
% of monocytes expressing GHR	95.2 (91.8, 97.7)	95.6 (92.3, 97.7)	0.9
% of neutrophils expressing GHR	92.8 (86.2, 97.7)	91.8 (85.5, 96.4)	0.7
Lymphocyte MFI	16.3 (12.9, 20.3)	17.3 (13.7, 21.8)	0.06
Monocyte MFI	17.1 (14.7, 19.9)	19.5 (16.8, 22.9)	0.2
Neutrophil MFI	12.9 (10.1, 16.8)	12.2 (9.5, 15.8)	0.2

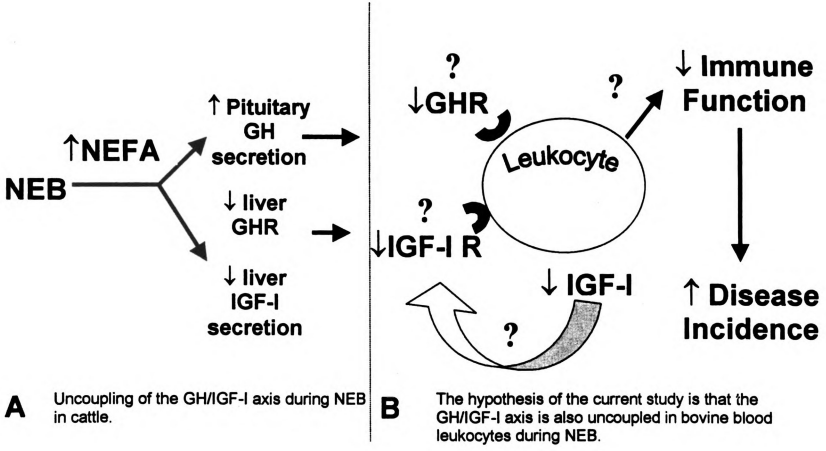


Figure 2.1.
Known (A) and proposed (B) effects of negative energy balance (NEB) on the growth hormone (GH)/ insulin-like growth factor I (IGF-I) axis. NEB causes an increase in serum non-esterified fatty acids (NEFA), an increase in blood GH and a decrease in blood IGF-I. This is partly due to decreased expression of the GH receptor (GHR) on the liver.

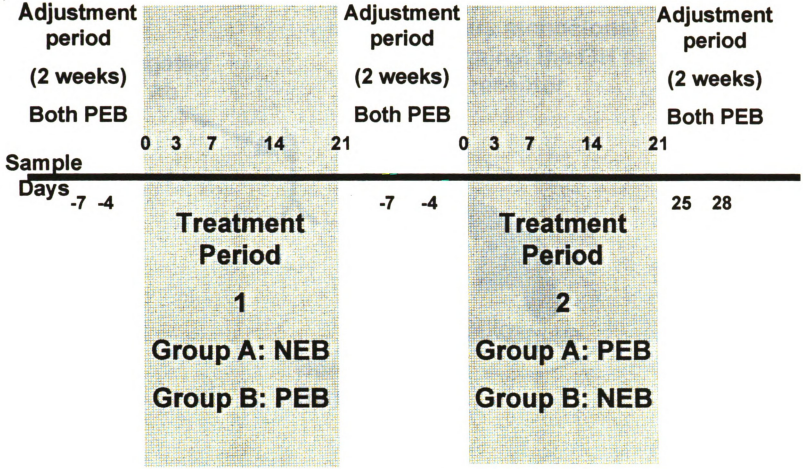


Figure 2.2. Model of the timeline of the trial. All steers were fed the positive energy balance (PEB) diet for 2 weeks before each treatment period. In treatment period 1, group A (8 steers) was fed the negative energy balance (NEB) diet and group B (8 steers) was fed the PEB diet. In treatment period 2 the treatments were reversed.

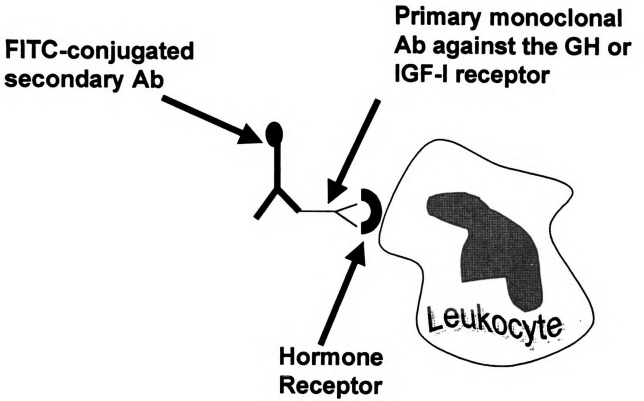


Figure 2.3. Indirect immunofluorescent staining of plasma membrane hormone receptors. (FITC= fluorescein isothiocyanate fluorochrome)

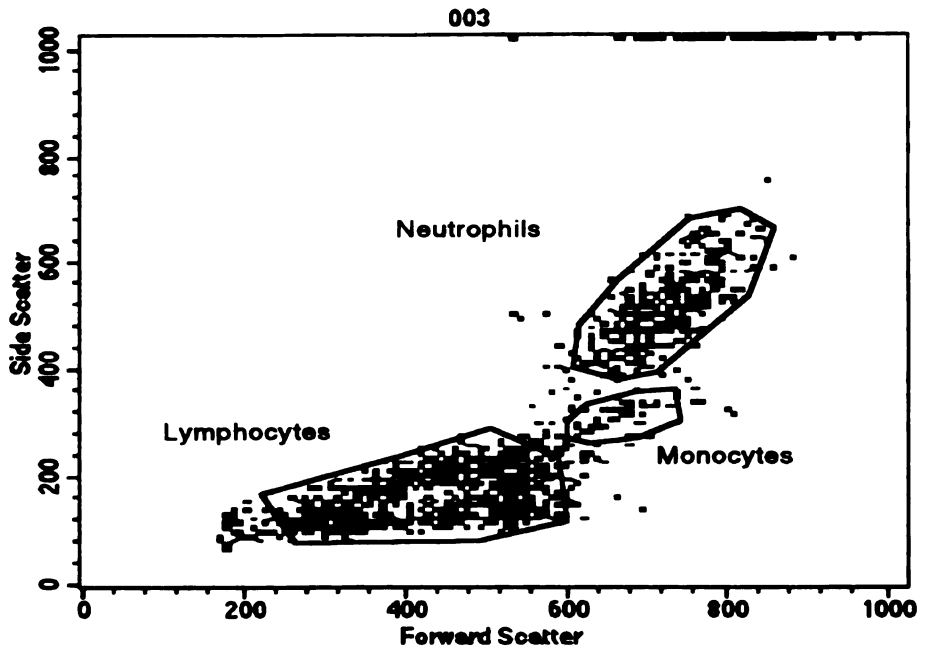


Figure 2.4. Density dot plot of physical characteristics (forward and side scatter) of bovine leukocytes. Data are from one representative steer.

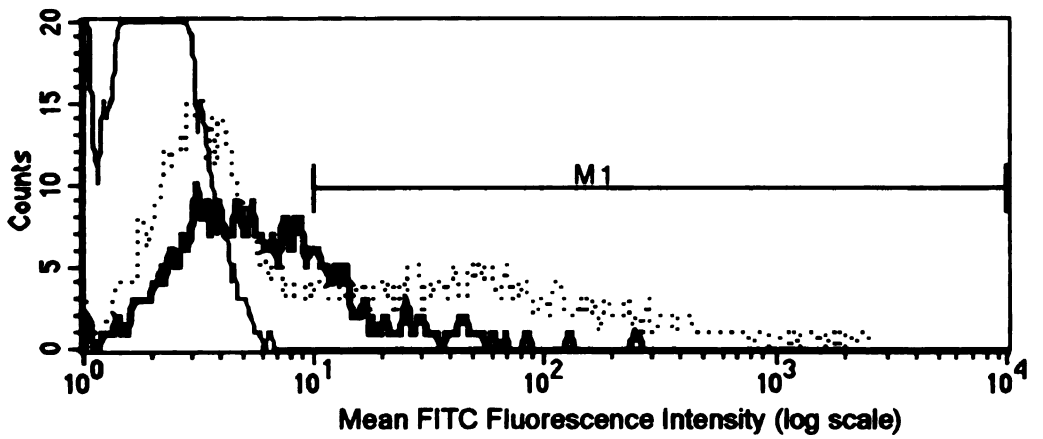


Figure 2.5. Histogram of mean fluorescence intensity (MFI) of lymphocytes immunostained for growth hormone receptor. Data are from one representative steer. Fluorescence during positive energy balance represented by the dashed histogram and negative energy balance by the solidhistogram. Events within M1 represent positive staining. Filled histogram represents negative isotype control.

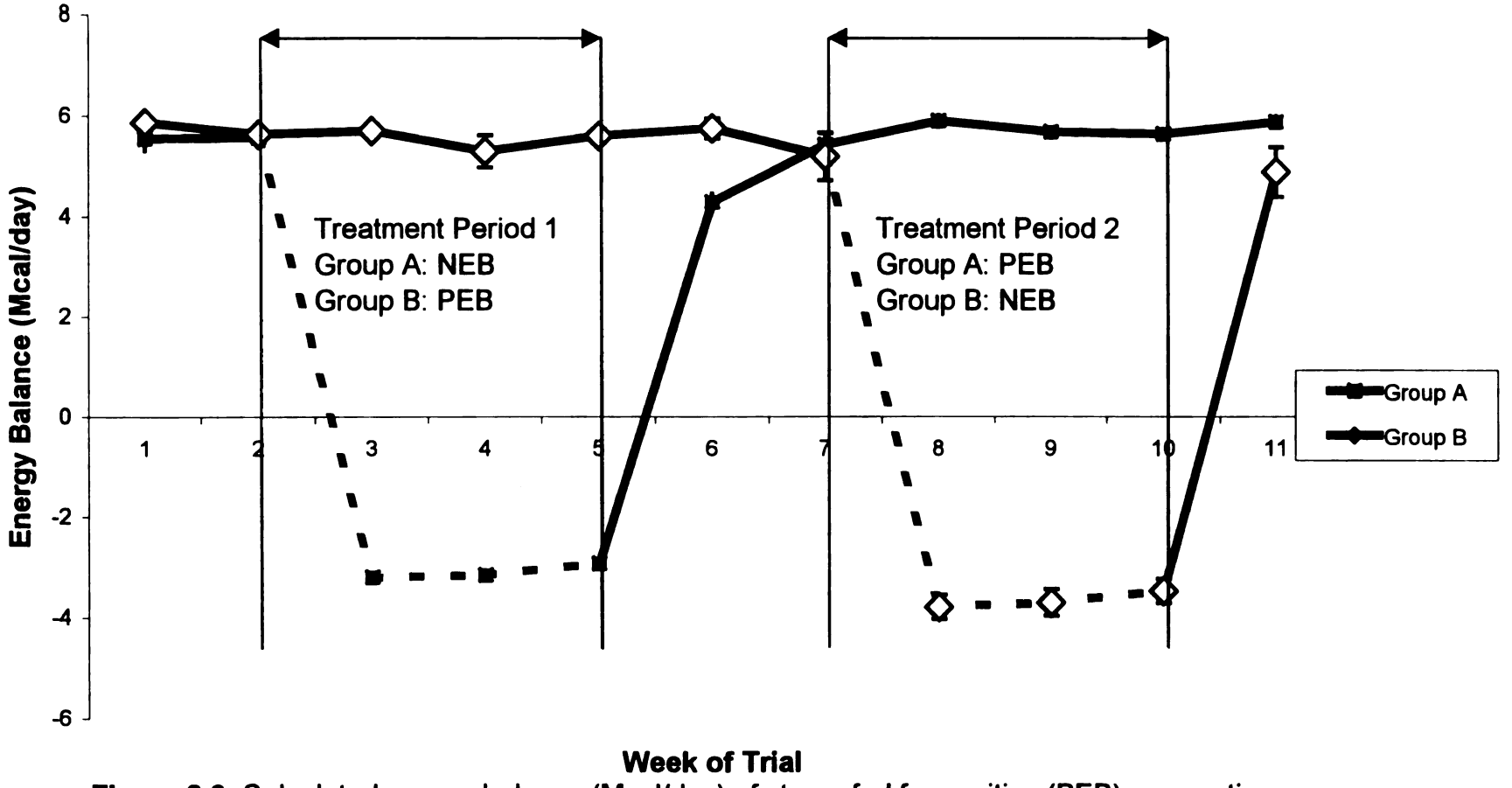


Figure 2.6. Calculated energy balance (Mcal/day) of steers fed for positive (PEB) or negative energy balance (NEB). Each line, squares or diamonds, follows one group through the entire trial. Lines are dashed when the steers of that group were fed the NEB diet and solid when the group was fed the PEB

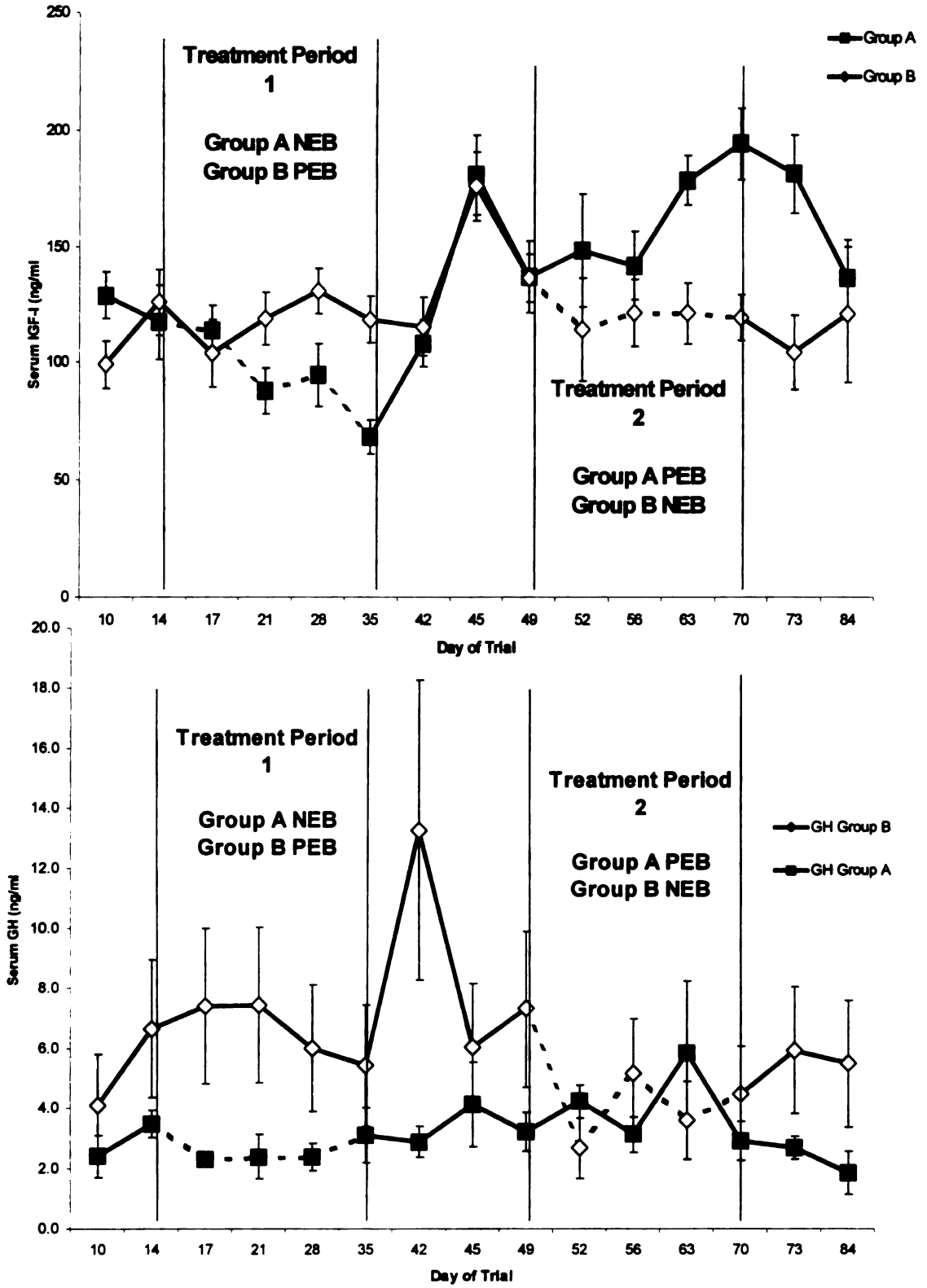


Figure 2.7. Circulating concentrations of growth hormone (GH) and insulin-like growth factor-I (IGF-I). Each line (squares or diamonds) follows one group through the trial. The line is dashed when steers of that group were fed the negative energy balance diet (NEB), and solid when they are fed the positive energy balance diet (PEB).

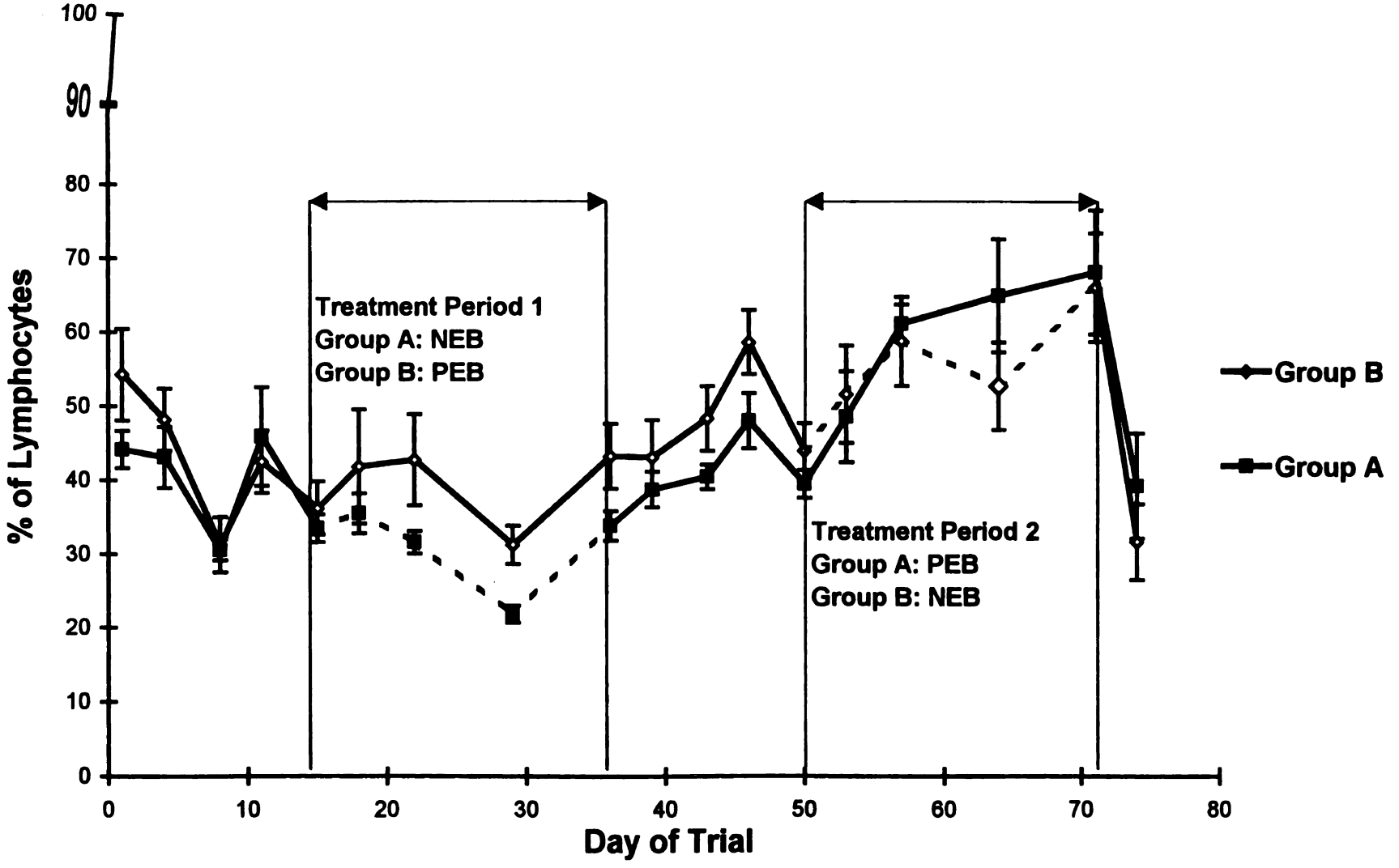


Figure 2.8. Percentage of lymphocytes expressing GH receptor in steers in positive (PEB) and negative energy balance (NEB) . Each line (squares or diamonds) follows one group through the entire trial. Lines are dashed when the steers of the group were fed the NEB diet and solid when the group was fed the PEB diet.

Chapter 3

EFFECT OF NEGATIVE ENERGY BALANCE ON GENE EXPRESSION OF L-SELECTIN (CD62L), AND MAC-1 (CD11B/CD18) BY BOVINE BLOOD LEUKOCYTES

Abstract

Sixteen yearling Holstein steers were fed for 210% or 60% of maintenance requirements to impose positive (PEB) or negative energy balance (NEB), respectively. Blood was collected, analyzed for serum concentration of nonesterified fatty acids (NEFAs), leukocytes were isolated and counted, and populations of neutrophils, lymphocytes and monocytes were gated out based on forward and side scatter characteristics using a flow cytometer. These populations were then analyzed for expression of the adhesion molecules L-selectin (CD62L), and Mac-1 (CD11b and CD18) using immunostaining and flow cytometric analysis. NEB increased the concentration of NEFA in serum ($P < 0.05$). Expression of CD62L on neutrophils was greater during NEB than PEB ($P = 0.03$). Energy balance did not affect expression of CD62L on any other cell types or expression of CD11b or CD18. The results of this study indicate that NEB does not affect negatively expression of CD62L, CD11b and CD18 by bovine blood leukocytes

Introduction

Adequate expression of the adhesion molecules L-selectin (CD62L), and Mac-1 (CD11b and CD18) is absolutely necessary for a successful immune response. These adhesion molecules facilitate adherence of leukocytes to the

endothelium and subsequent migration of leukocytes between endothelial cells into infected tissue. If any of these adhesion molecules are missing, leukocytes will be unable to leave the circulation and fight infection. For example, cattle with bovine leukocyte adhesion deficiency (BLAD) lack the CD18 molecule and usually die at a young age due to widespread opportunistic infection (Kehrli et al., 1990; Nagahata et al., 1994; 1997).

Expression of CD62L and CD18 decreases after parturition and may contribute to increased susceptibility to disease (Lee and Kehrli, 1998). One possible cause for the decreased expression of these molecules may be malnutrition. Typically, the dairy cow is in NEB around the time of parturition due to increased demand for energy and decreased dry matter intake. To compensate, the dairy cow mobilizes body reserves in the form of plasma NEFA (Grummer, 1995; Grum et al., 1996). A study of cows in commercial farms showed that cows with high concentration of NEFAs in plasma prepartum had an increased incidence of mastitis after calving (Dyk et al., 1995). This relationship between NEFA and mastitis suggests that a shortage of energy might be partly responsible for the increased susceptibility to disease seen in the periparturient period. This is further supported by the finding that children suffering from protein-calorie malnutrition have decreased neutrophil function (Chandra, 1992; 1997). The hypothesis of this study was that NEB impairs neutrophil expression of the adhesion molecules CD62L, CD18 and CD11b (Figure 3.1). The objective of the current study was to determine the effect of NEB on expression of CD62L, CD18 and CD11b by bovine blood leukocytes.

Materials and Methods

Unless otherwise specified, methods are as described in Chapter 2.

A. CD62L, CD11b, and CD18 Immunostaining Procedure

Prepared leukocytes (Chapter 2) were centrifuged at 350 x *g* for 5 min at 22°C. Supernatant was aspirated and primary monoclonal antibodies were added. The antibodies used were anti-CD62L IgG1 (clone BAQ92A from VMRD, Pullman WA), anti-CD11b IgG1 (clone MM12A from VMRD, Pullman, WA), and anti-CD18 IgG1 (clone BAQ30A from VMRD, Pullman WA). All three primary antibodies were diluted to 7μl /ml with wash solution. One well per steer received 100μl of the anti-CD62L dilution; one well per steer received 100μl of the anti-CD11b dilution; and one well per steer received 100μl of the anti-CD18 IgG1 dilution. Plates were incubated in the dark at 4°C for 30 min and then centrifuged at 350 x *g* for 5 min at 22°C. Supernatant was aspirated and cells were washed with 200 μl of wash solution. Secondary antibody was then added. The same secondary antibody was added (100 μl) to all primary antibodies and was conjugated to phycoerythrin (PE; Figure 3.2). The secondary antibody was anti-mouse IgG1 diluted to a final concentration of 1:100 with wash solution (clone M32004 from Caltag, San Francisco, CA). Irrelevant mouse IgG1 PE Isotype Control diluted to 7μl /ml with wash solution (50 μl/well; clone X0927 from Dako, Carpinteria, CA) was added to one well per steer and served as the negative control. Plates were incubated in the dark at 4°C for 15 min and then centrifuged at 350 x *g* for 5 min at 22°C. Supernatant was aspirated and cells were fixed with a 1:10 dilution (in H_2O) of FACS Lysing Solution (150 μl/well,

Becton Dickinson, San Jose, CA) for 10 min in the dark at 22°C to stop the metabolic functions of the cells. Plates were centrifuged again, supernatant was aspirated, cells were washed one final time in 200 μl wash solution, and then cells were resuspended in 200 μl of sheath fluid (Isoton II, Coulter Diagnostics, Hialeah, FL) for immediate flow cytometric analysis.

B. Flow Cytometric Analysis of CD62L, CD18 and CD11b

The FACSCalibur fluorescence activated flow cytometer and the acquisition package of CellQuest software (Becton Dickinson, San Jose, CA) were used to acquire and analyze the leukocyte adhesion molecule data. Unstained cells in 2 wells per steer were used to check the forward scatter (FSC = size characteristics) and side scatter (SSC = granularity characteristics) parameters of the leukocytes. These parameters allowed individual animal variation in physical leukocyte characteristics to be adjusted in FSC-SSC density dot plots (Figure 2.4). Detectors for PE fluorescence were adjusted in preliminary experiments for optimal demonstration of red fluorescence. Detector settings were not altered during the experiment. Data from 5000 events (approximately 5000 cells) per well were acquired.

For expression of CD62L, neutrophils, monocytes, and lymphocytes were gated out based on side scatter (granularity) and PE fluorescence characteristics (Figure 3.3a).

For expression of CD18, four cell types were gated out based on SSC (granularity) and PE fluorescence characteristics. These populations were neutrophils, monocytes, and two distinct populations of lymphocytes. One

population of lymphocytes stained more brightly than the other and is labeled "bright lymphocytes (Figure 3.3b)."

For expression of CD11b, four cell populations were gated out based on SSC (granularity) and PE fluorescence characteristics. These populations included neutrophils, monocytes, a small population of positively stained lymphocytes and a larger population of unstained lymphocytes (Figure3.3c).

The PE fluorescence histograms of cells in each gated region were then plotted (Figure 3.4a,b,c) and geometric mean fluorescence intensities (MFI) were computed from the Y-axis (cell counts) and the X-axis (intensity channel). The region of background fluorescence based on the negative control was identified (solid green peak in Figure 3.4abc) and events to the right of a marker (M1) were considered positive fluorescence events (Figure 3.4abc). The position of M1 was not altered throughout the duration of the trial so that any shift in positive fluorescence intensity over time (treatment effect) was detectable.

C. Leukocyte Counts

Leukocytes were counted as described in Chapter 2.

D. Statistical Analyses

Specific questions were asked to aid in the development of appropriate statistical models. Questions are as follows:

5) is there a mean difference between NEB and PEB in treatment period 1;

6) is there a mean difference between NEB and PEB in treatment period 2;

7) is there a mean carryover effect from treatment period 1 into treatment period 2; and,

8) is there an overall mean difference between PEB and NEB?

Statistical Analysis Software (SAS® PROC MIXED) was used to analyze data by repeated measures analysis correcting for correlated error within steer (SAS®, 1996). The linear model included fixed effects of group, day (relative to the start of the trial), and plate (flow cytometric data were acquired using two 96-well microtiter plates), the interactions of group by day, plate by day, and plate by group (Model 1). Repeated measures within animal were modeled as a heterogeneous compound symmetry structure such that the random animal effect was fitted indirectly and an allowance was made for unequal residual variances across days (Littell et al., 1998). The test term (error) for group, plate, and group by plate was animal within group by plate. Any model term involving day was tested against the residual variance. Treatment was indirectly fitted through group and group by day model terms. The model (Model 2) for leukocyte counts, energy balance, body weight, and NEFA did not include plate or interactions involving plate. These data, along with MFI data were log transformed because of erratic heterogeneous variance observed upon initial examination of empirical distribution of the residuals.

Contrasts were derived to provide estimates for the treatment effects in order to address the questions described previously. This was done by using the following equations in which $\mu_{i,j}$ represents the LSM of the *i*th group on the *j*th

day of the study. For example $\mu_{A,28}$ represents the least squares mean (LSM) of group A on day 28 relative to the start of the trial (refer to Figure 2.2).

In order to address the first two questions, the average PEB and NEB values of each dependent variable for each treatment period were estimated. These values were then contrasted as follows:

$\frac{1}{2}$ $(\mu_{A,28} + \mu_{A,35}) - \frac{1}{2}$ $(\mu_{B,28} + \mu_{B,35})$ = estimated difference between NEB (group A) and PEB (group B) in treatment period 1; and,

$\frac{1}{2}$ $(\mu_{A,63} + \mu_{A,70}) - \frac{1}{2}$ $(\mu_{B,63} + \mu_{B,70})$ = estimated difference between PEB (group A) and NEB (group B) in treatment period 2.

To determine if carryover effects were present, thereby answering question three, similar procedures were followed for data obtained during the adjustment period when both groups were in PEB. Individual estimates of the difference in the dependent variable of the two groups were calculated. Estimates were determined for day 39, 43, and 46 using the following contrasts:

$\mu_{A,39} - \mu_{B,39}$ = estimated difference between group A and group B on day 39;

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 43; and,

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 46.

The following contrast also was developed to compare the LSM for each group across the 3 sample days during the adjustment period:

1/3 ($\mu_{A,39} + \mu_{A,43} + \mu_{A,46}$) – 1/3 ($\mu_{B,39} + \mu_{B,43} + \mu_{B,46}$) = estimated difference between group A and group B across days 39, 43 and 46.

The results of these contrasts showed that some of the dependent variables measured (GH receptor, CD18, CD11b, MHC class I, and plasma GH pre-feeding) were different between the two groups on day 46. In order to simplify analyses, all data were corrected for this carryover when estimating the overall difference between PEB and NEB.

To correct for carryover effects and therefore answer question four, the LSM for the last two samples of each treatment period (day 28, 35 and 63, 70) for each group (A or B) were averaged as follows:

A: ½ ($\mu_{A,28} + \mu_{A,35}$) = estimated LSM for group A, treatment period 1(NEB);

B: ½ ($\mu_{A,63} + \mu_{A,70}$) = estimated LSM for group A, treatment period 2(PEB);

C: ½ ($\mu_{B,28} + \mu_{B,35}$) = estimated LSM for group B, treatment period 1 (PEB); and,

D: ½ ($\mu_{B,63} + \mu_{B,70}$) = estimated LSM for group B, treatment period 2 (NEB).

This resulted in two treatment means per group, one for each treatment period or one PEB mean and one NEB mean. Similarly, the average of the LSM from the two samples prior to the start of the each treatment period (day 10, 14 and 46, 50) for each group were calculated:

E: ½ ($\mu_{A,10} + \mu_{A,14}$) = estimated LSM for group A, pre-treatment period 1 (pre-NEB);

F: ½ ($\mu_{A,46} + \mu_{A,50}$) = estimated LSM for group A, pre-treatment period 2 (pre-PEB);

G: ½ ($\mu_{B,10}$ + $\mu_{B,14}$) = estimated LSM for group B, pre-treatment period 1 (pre-PEB); and,

H: ½ ($\mu_{B,46}$ + $\mu_{B,50}$) = estimated LSM for group B, pre-treatment period 2 (pre-NEB).

These averages were combined in a contrast that corrected the LSM of the treatment periods for pre-treatment values:

½ (A - E) + ½ (D - H) = estimate of overall NEB adjusted for pre-treatment means;

½ (C - G) + ½ (B - F) = estimate of overall PEB adjusted for pre-treatment means; and,

[½ (A - E) + ½ (D - H)] – [½ (C - G) + ½ (B - F)] = estimated overall difference between NEB and PEB adjusted for pre-treatment means.

Data are presented as LSM with 95% upper and lower confidence intervals and were backtransformed to be presented in original units.

Model 1

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \alpha\beta_{ij} + \alpha\gamma_{ik} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *k*th plate and the *l*th steer. The dependent variables monitored included:

mean fluorescence intensity of CD62L, CD18 and CD11b staining in lymphocytes, monocytes and neutrophils.

μ = overall mean

α_i = fixed effect of group (*i* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

γ_k = fixed effect of plate (*k* = 1,2)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

$\alpha\gamma_{ik}$= interaction of *i*th group and *k*th plate

$\beta\gamma_{jk}$= interaction of *j*th day and *k*th plate

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all *i,j,k,l* and equally correlated across time within the *l*th steer, allowing for heterogeneity in variance over time (i.e., the repeated measures option of SAS PROC MIXED, Type=CSH).

Model 2

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *k*th steer. The dependent variables monitored included:
serum NEFA;
body weight;
calculated energy balance;
Total circulating leukocytes
circulating lymphocytes;
circulating monocytes; and,
circulating neutrophils.

μ = overall mean

α_i = fixed effect of group (*i* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all *i,j,k,l* and equally correlated across time within the *k*th steer, allowing for heterogeneity in variance over time (i.e., the repeated measures option of SAS PROC MIXED, Type=CSH).

Results

A. Energy Balance

Results are as described in Chapter 2.

B. Leukocyte Counts

Results are as described in Chapter 2.

C. Expression of CD62L, CD11b and CD18

The percentages of cells expressing these molecules were not analyzed because it was approximately 100% for all leukocytes. Energy balance did not alter MFI of CD11b and CD18 in any leukocyte population. There also was no

effect of NEB on MFI of CD62L in lymphocytes and monocytes. However, we observed a slight increase in MFI of CD62L expression by bovine blood neutrophils ($P = 0.03$).

Discussion

Dairy cows are immunosuppressed around the time of parturition (Kehrli et al., 1989a,b; Lee and Kehrli, 1998; Detilleux et al., 1995). Neutrophils from periparturient cows demonstrate dysfunctional migration, chemotaxis and decreased production of reactive oxygen species (Kehrli et al., 1989b; Nagahata et al., 1988; Saad et al., 1989). Furthermore, periparturient cows have decreased expression of CD62L and CD18, which may contribute to altered migration and increased susceptibility to disease (Lee and Kehrli, 1998).

The current study showed for the first time that NEB in cattle did not affect negatively the expression of the adhesion molecules CD62L, CD11b, and CD18 by unstimulated blood leukocytes. Neutrophil migration is initially dependent on CD62L. CD62L is expressed constitutively on resting neutrophils and is rapidly down-regulated in response to chemotactic signals, such as platelet activating factor, present at sites of inflammation or tissue injury. Glucocorticoids also are known to cause down-regulation of CD62L (Burton et al.,1995). For example, treatment of dairy bulls with dexamethasone induced down-regulation of CD62L within 8 hours of injection (Burton et al., 1995). Binding of CD62L to the endothelium and chemoattractant release from endothelial cells stops leukocyte rolling by enhancing expression of Mac-1 (CD11b/CD18). This molecule allows

the leukocyte to tightly adhere to the endothelium and migrate into the tissue along a chemoattractant gradient (Figure 1.4; Adams and Shaw, 1994).

Expression of adhesion molecules is altered around the time of parturition. Expression of CD62L decreases gradually for 2 weeks prepartum and decreases markedly on the day of calving (Lee and Kehrli, 1998). Expression of CD18 is high before calving and peaks at calving, but decreases by 15 hours postpartum (Lee and Kehrli, 1998). In the current study, there was no change in MFI during NEB for CD11b or CD18 and a slight increase in MFI of CD62L on neutrophils during NEB. These data suggest that NEB is not involved in the decreased expression of adhesion molecules in this steer model in which NEB was caused by metabolizable energy restriction.

However, this study did not include any experimental immune challenge. Furthermore, the leukocytes analyzed were all from the circulating pool. Thus the cells were supposedly resting and had no stimulus (e.g., infection or inflammation) for altering expression of adhesion molecules. In an unstimulated leukocyte, CD11b and CD18 are stored in granules inside the cell. These molecules are only expressed in response to chemoattractants such as platelet-activating factor released during inflammation (Gilbert et al., 1992). For example, when the mammary gland becomes infected in mastitis, the inflammatory response releases platelet-activating factor from the endothelium. This causes neutrophils that are expressing CD62L and rolling along the endothelium to up-regulate CD11b and CD18 and shed CD62L. The neutrophil then tightly adheres to the endothelium and enters the mammary tissue to fight the infection. The

observed increase in CD62L expression in this study may indicate a problem with the ability of neutrophils to shed CD62L in response to inflammatory stimuli. If CD62L cannot be shed, the neutrophil will be unable to enter the infected tissue and immunocompetence becomes impaired.

The use of steers in this study was intended to prevent the interaction of NEB with these endocrine changes with the exception of GH and IGF-I. However, in so doing, the right combination of endocrine changes needed for decreased immunocompetence may not have been included. Therefore, this study may need to be done in periparturient dairy cows.

Further studies also should be conducted to determine if NEB in cattle impairs expression of these molecules in response to an inflammatory stimulus. Nonetheless, the current findings certainly challenge the conventional theory that NEB plays a major role in the immunosuppression associated with calving.

Chapter Three

Figures

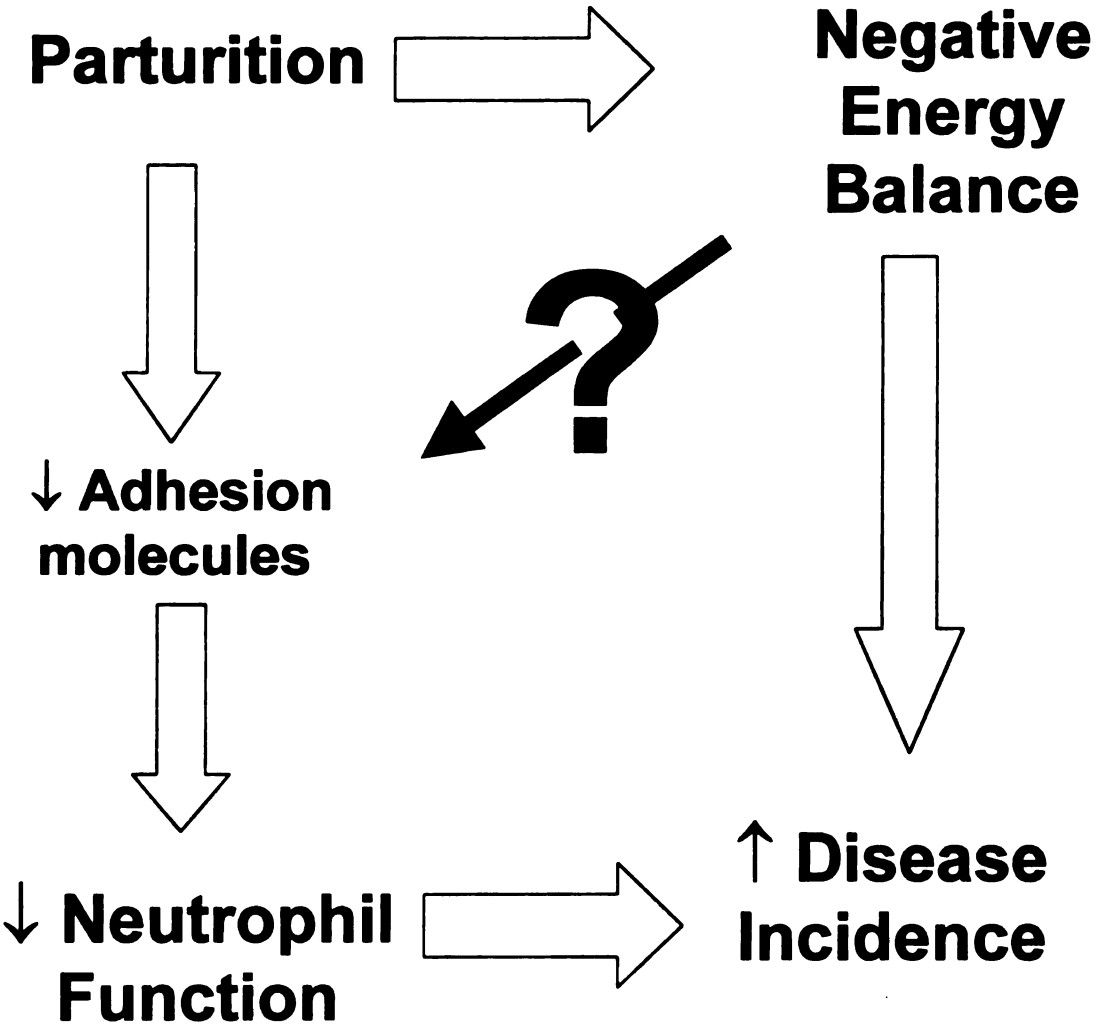


Figure 3.1. Proposed model of the hypothesis that negative energy balance (NEB) impairs leukocyte expression of the adhesion molecules CD62L, CD11b, and CD18.

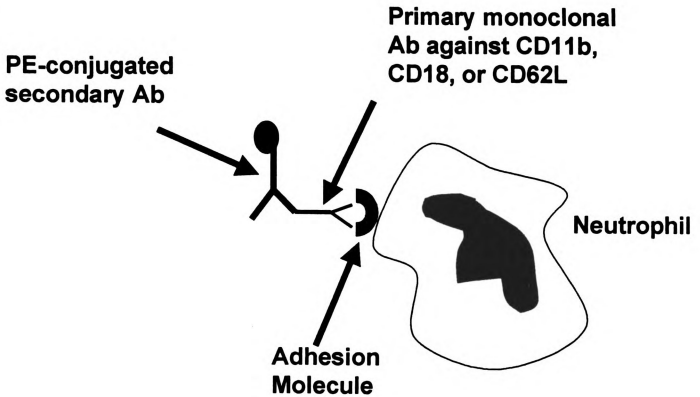
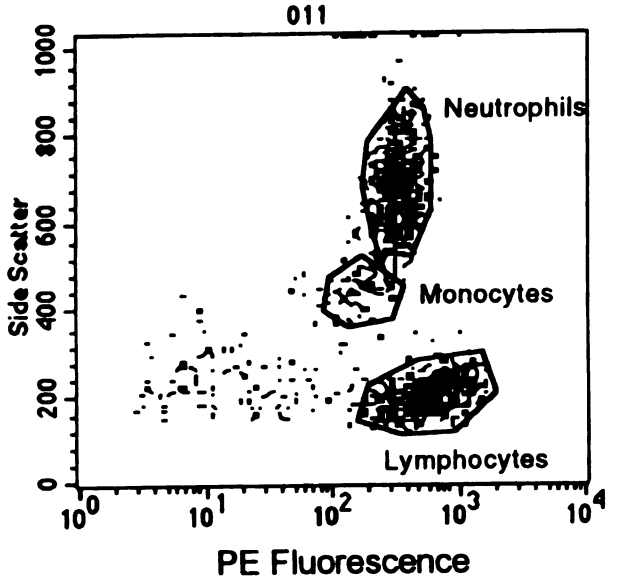


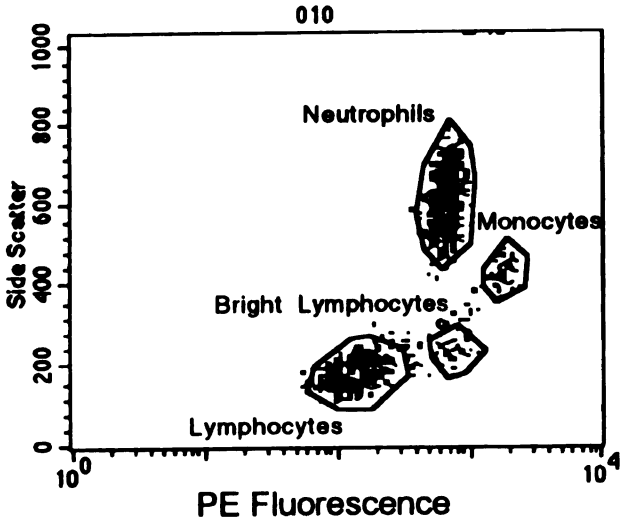
Figure 3.2. Indirect immunofluorescent staining of adhesion molecules CD62L, CD18, and CD11b. (PE = phycoerythrin fluorochrome).

Figure 3.3. Flow cytometric identification for a representative animal of A) CD62L, B) CD18, and C) CD11b differentiated bovine leukocyte populations based on PE fluorescence (log scale; x-axis) and side scatter characteristics (granularity; y-axis). In A) three populations were identified, neutrophils, monocytes and lymphocytes. In B) four populations were identified, neutrophils, monocytes, lymphocytes, and a subset of lymphocytes that stained more brightly or "bright lymphocytes." In C) four populations were identified, neutrophils, monocytes, positively stained lymphocytes and negatively stained lymphocytes.

A.


011
Neutrophils
Monocytes
Lymphocytes
Side Scatter
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
PE Fluorescence

B.


010
Neutrophils
Monocytes
Bright Lymphocytes
Lymphocytes
Side Scatter
0
200
400
600
800
1000
10^0
10^4
PE Fluorescence

C.

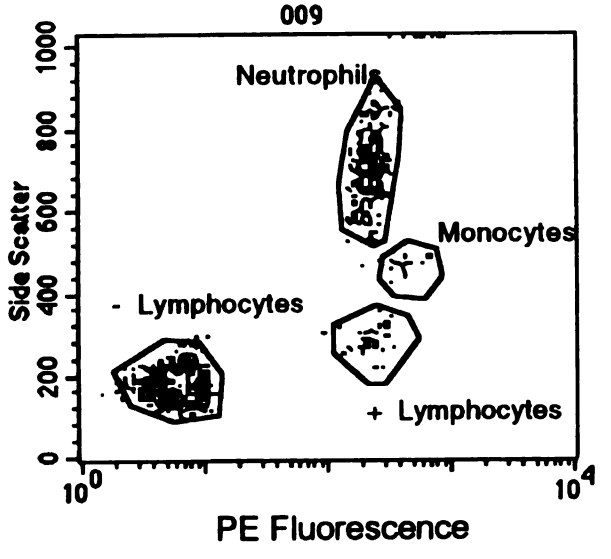
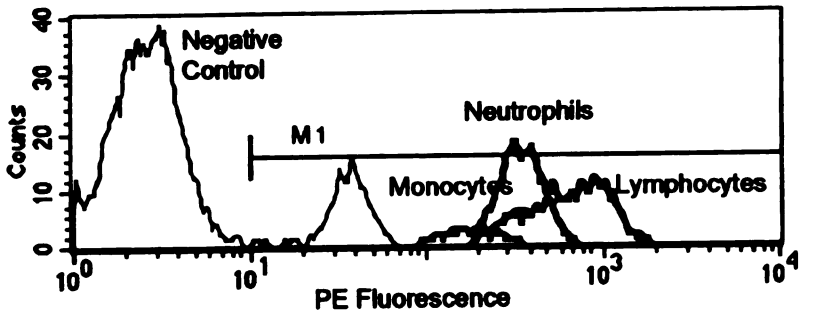
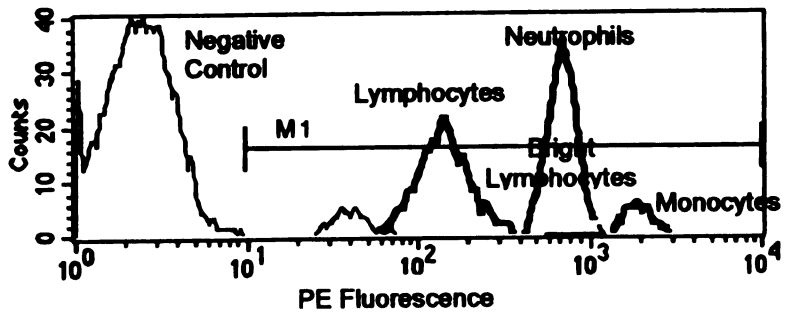

009
Neutrophils
Monocytes
- Lymphocytes
+ Lymphocytes
Side Scatter
0
200
400
600
800
1000
10^0
10^4
PE Fluorescence

Figure 3.4. Differential expression of A) CD62L, B) CD18, and C) CD11b in PE stained bovine leukocytes was determined by PE-mean fluorescence intensity (x-axis). Shown in the histograms are representative peaks for each leukocyte type from a representative animal. In all three histograms, the solid green peak represents the negative control, the red peak represents neutrophils, and the purple peak represents monocytes. In B) the blue peak represents lymphocytes and the orange peak represents brightly stained lymphocytes. In C) the blue peak represents positively stained lymphocytes and the orange peak represents lymphocytes that did not stain for CD11b.

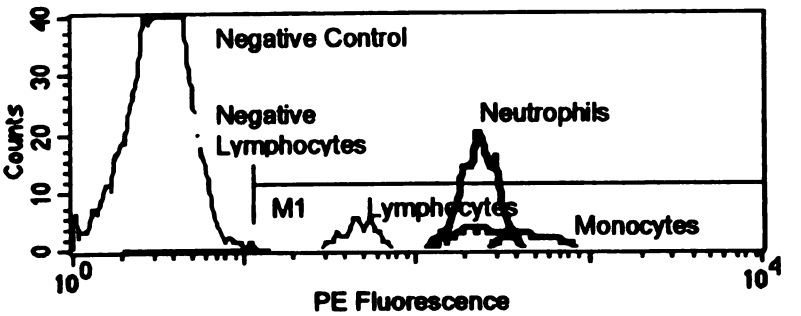
A.
CD62L



B.
CD18



C.
CD11b



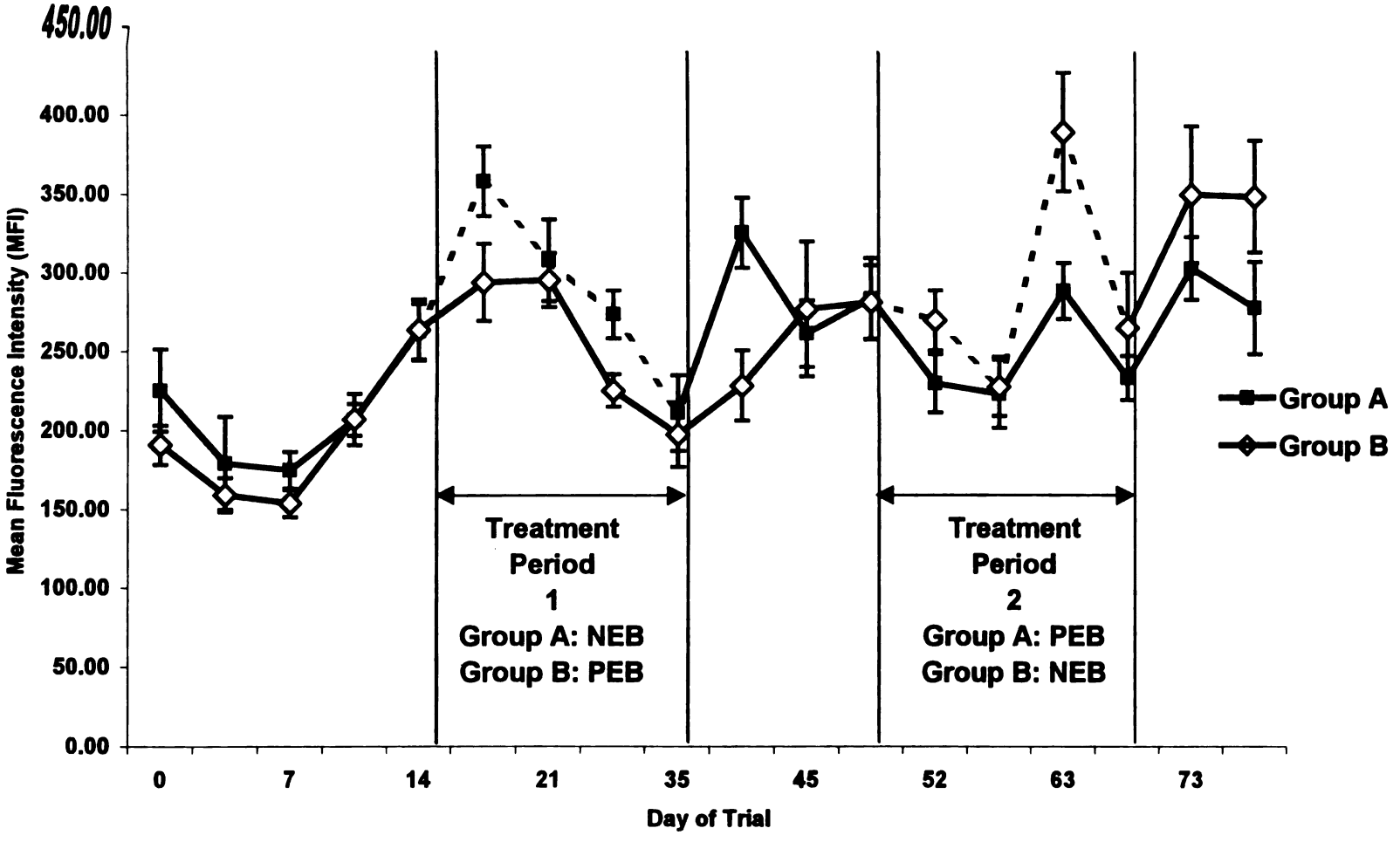


Figure 3.5. Mean fluorescence intensity (MFI) of expression of CD62L by blood neutrophils from steer in positive (PEB) or negative energy balance (NEB). Each line (squares or diamonds) follows one group through the entire trial. Lines are dashed when steers of the group were fed the NEB diet and solid when

Chapter 4

EFFECT OF NEGATIVE ENERGY BALANCE ON GENE EXPRESSION OF MAJOR HISTOCOMPATABILITY COMPLEX (MHC) CLASS I AND CLASS II BY BOVINE BLOOD LEUKOCYTES

Abstract

Sixteen yearling Holstein steers were fed for 210% or 60% of maintenance requirements to impose positive (PEB) or negative energy balance (NEB), respectively. Blood was collected and analyzed for serum concentration of nonesterified fatty acids (NEFA), and leukocytes were isolated and analyzed for expression of major histocompatability complex (MHC) class I and class II molecules using immunostaining and flow cytometric analysis. NEB increased circulating concentration of NEFA ($P < 0.05$). NEB did not affect MHC class I expression, but resulted in a small but significant increase in the expression of MHC class II ($P = 0.03$). I conclude that nutritionally created NEB does not impair expression of MHC class I or MHC class II by bovine blood leukocytes.

Introduction

Effective immune function depends upon the interaction and balance among several different leukocyte types. Especially important are the interactions of subsets of lymphocytes, which are mediated in part by MHC expression (Figure 1.2; Van Kampen and Mallard, 1997).

Around the time of parturition, the dairy cow is immunosuppressed (Table 1.2; Detilleux et al., 1995; Kehrli and Goff, 1989; Kehrli et al., 1989a,b). This immunosuppression may be caused in part by various metabolic and physical

challenges via effects on the neuro-endocrine-immune axis. One such challenge may be protein-calorie malnutrition, a large component of which is negative energy balance (NEB). The dairy cow faces an enormous increase in the demand for energy by the onset of lactation at calving. This is further compounded by a decrease in feed intake (VandeHaar et al., 1999; Grummer, 1995). To compensate, the cow mobilizes body reserves in the form of non-esterified fatty acids (NEFA). A study on commercial dairy farms found that cows with high serum NEFA prepartum had an increased incidence of mastitis and retained placenta postpartum (Dyk et al., 1995). Furthermore, populations of leukocytes expressing MHC class II are highest before calving but decrease immediately after parturition (Van Kampen and Mallard, 1997). Malnutrition in pigs alters MHC class I and II expression thereby increasing susceptibility to, and duration of, infection (McCracken et al., 1999; Zijlstra et al., 1999). These studies all suggest that malnutrition may alter MHC expression. The hypothesis of this study is that NEB in cattle decreases expression of MHC class I and class II by bovine leukocytes. The objectives were to monitor the expression of MHC class I and II during experimentally induced NEB.

Materials and Methods

Unless otherwise specified the methods are as described in Chapter 2.

A. MHC Class I and MHC Class II Immunostaining Procedure

Prepared leukocytes (Chapter 2) were centrifuged at 350 x *g* for 5 min at 22°C, supernatant was aspirated and primary monoclonal antibodies were added. The antibodies used were anti-MHC class I IgG2a (clone H58A from

VMRD, Pullman WA), and anti-MHC class II IgG1 (clone CAT82A from VMRD, Pullman, WA).

One well per steer received 100 µl of a 3.5 µl/ml dilution (in wash solution) of anti-MHC class I, and one well per steer received 100 µl of a 7 µl/ml dilution (in wash solution) of anti-MHC class II. Plates were incubated in the dark at 4°C for 30 min and then centrifuged at 360 x *g* for 5 min at 22°C. Supernatant was aspirated and cells were washed with 200 µl of wash solution. Secondary antibodies were then added. Both secondary antibodies were conjugated to phycoerythrin (PE; Figure 3.2) and 100 µl was added to the appropriate primary antibody. The secondary antibody for MHC class I was a 1:200 dilution of anti-mouse IgG2a-PE (clone M32204 from Caltag, San Francisco, CA). The secondary antibody for MHC class II was a 1:100 dilution of anti- IgG1-PE (clone M32004 from Caltag, San Francisco, CA). A 7µl/ml dilution (in wash solution) of irrelevant mouse IgG1 PE Isotype Control (0.35 µl/well ; clone X0928 from Dako, Carpinteria, CA) was added to one well per steer and served as the negative control. Plates were incubated in the dark at 4°C for 15 min then centrifuged at 350 x *g* for 5 min at 22°C. Supernatant was aspirated and cells were fixed with a 1:10 dilution (in ddH_2O) of FACS Lysing Solution (15 µl/well, Becton Dickinson, San Jose, CA) for 10 min in the dark at 22°C to stop the metabolic functions of the cells. Plates were centrifuged again, supernatant was aspirated, cells were washed one final time in 200 µl wash solution and the cells were then resuspended in 200 µl of sheath fluid (Isoton II, Coulter Diagnostics, Hialeah, FL) for immediate flow cytometric analysis.

B. Flow Cytometric Analysis of MHC Class I and Class II

The FACSCalibur fluorescence-activated flow cytometer and the acquisition package of CellQuest software (Becton Dickinson, San Jose, CA) were used to acquire and analyze the leukocyte MHC class I and class II data. Unstained cells in 2 wells per steer were used to check the forward scatter (FSC = size characteristics) and side scatter (SSC = granularity characteristics) parameters of the leukocytes. These parameters allowed individual animal variation in physical leukocyte characteristics to be adjusted in FSC-SSC density dot plots (Figure 2.4). Detectors for PE fluorescence were adjusted in preliminary experiments for optimal demonstration of red fluorescence. Detector settings were not altered during the experiment. Data from 5000 events (approximately 5000 cells) per well were acquired.

For expression of MHC class I, neutrophils, monocytes, and lymphocytes were gated out based on side scatter (granularity) characteristics and PE fluorescence on density dot plots (Figure 4.1a).

For expression of MHC class II, one cell population was gated out based on SSC characteristics and FL-2 (PE) fluorescence. This population was a small positively stained group of mononuclear cells (Figure 4.1b).

The PE fluorescence histograms of cells in each gated region were then plotted (Figure 4.2ab) and geometric mean fluorescence intensities (MFIs) were computed from the Y-axis (cell counts) and the X-axis (intensity channel). The region of background fluorescence based on the negative control was identified (solid green peak in Figure 4.2ab) and events to the right of a marker (M1) were

considered positive fluorescence events (Figure 4.2ab). The percentage of gated cells that fell within this positive region was also determined for MHC class II stained cells. The position of M1 was not altered throughout the duration of the trial so that any shift in positive fluorescence intensity over time (treatment effect) was detectable.

H. Statistical Analyses

Specific questions were asked to aid in the development of appropriate statistical models. Questions are as follows:

9) is there a mean difference between NEB and PEB in treatment period 1;

10) is there a mean difference between NEB and PEB in treatment period 2;

11) is there a mean carryover effect from treatment period 1 into treatment period 2; and,

12) is there an overall mean difference between PEB and NEB?

Statistical Analysis Software (SAS® PROC MIXED) was used to analyze data by repeated measures analysis correcting for correlated error within steer (SAS®, 1996). The linear model included fixed effects of group, day (relative to the start of the trial), and plate (flow cytometric data were acquired using two 96-well microtiter plates), the interactions of group by day, plate by day, and plate by group (Model 1). Repeated measures within animal were modeled as a heterogeneous compound symmetry structure such that the random animal effect was fitted indirectly and an allowance was made for unequal residual

variances across days (Littell et al., 1998). The test term (error) for group, plate, and group by plate was animal within group by plate. Any model term involving day was tested against the residual variance. Treatment was indirectly fitted through group and group by day model terms. The model (Model 2) for leukocyte counts, energy balance, body weight, and NEFA did not include plate or interactions involving plate. These data, along with MFI data were log transformed because of erratic heterogeneous variance observed upon initial examination of empirical distribution of the residuals. All percentage data were converted to decimal values and transformed as the inverse sine of the square root.

Contrasts were derived to provide estimates for the treatment effects in order to address the questions described previously. This was done by using the following equations in which μ_{ij} represents the LSM of the ith group on the jth day of the study. For example $\mu_{A,28}$ represents the least squares mean (LSM) of group A on day 28 relative to the start of the trial (refer to Figure 2.2).

In order to address the first two questions, the average PEB and NEB values of each dependent variable for each treatment period were estimated. These values were then contrasted as follows:

$\frac{1}{2}(\mu_{A,28} + \mu_{A,35}) - \frac{1}{2}(\mu_{B,28} + \mu_{B,35})$ = estimated difference between NEB (group A) and PEB (group B) in treatment period 1; and,

$\frac{1}{2}(\mu_{A,63} + \mu_{A,70}) - \frac{1}{2}(\mu_{B,63} + \mu_{B,70})$ = estimated difference between PEB (group A) and NEB (group B) in treatment period 2.

To determine if carryover effects were present, thereby answering question three, similar procedures were followed for data obtained during the adjustment period when both groups were in PEB. Individual estimates of the difference in the dependent variable of the two groups were calculated. Estimates were determined for day 39, 43, and 46 using the following contrasts:

$\mu_{A,39} - \mu_{B,39}$ = estimated difference between group A and group B on day 39;

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 43; and,

$\mu_{A,43} - \mu_{B,43}$ = estimated difference between group A and group B on day 46.

The following contrast also was developed to compare the LSM for each group across the 3 sample days during the adjustment period:

$1/3\ (\mu_{A,39} + \mu_{A,43} + \mu_{A,46}) - 1/3\ (\mu_{B,39} + \mu_{B,43} + \mu_{B,46})$ = estimated difference between group A and group B across days 39, 43 and 46.

The results of these contrasts showed that some of the dependent variables measured (GH receptor, CD18, CD11b, MHC class I, and plasma GH pre-feeding) were different between the two groups on day 46. In order to simplify analyses, all data were corrected for this carryover when estimating the overall difference between PEB and NEB.

To correct for carryover effects and therefore answer question four, the LSM for the last two samples of each treatment period (day 28, 35 and 63, 70) for each group (A or B) were averaged as follows:

A: ½ ($\mu_{A,28}$ + $\mu_{A,35}$) = estimated LSM for group A, treatment period 1(NEB);

B: ½ ($\mu_{A,63}$ + $\mu_{A,70}$) = estimated LSM for group A, treatment period 2(PEB);

C: ½ ($\mu_{B,28}$ + $\mu_{B,35}$) = estimated LSM for group B, treatment period 1 (PEB); and,

D: ½ ($\mu_{B,63}$ + $\mu_{B,70}$) = estimated LSM for group B, treatment period 2 (NEB).

This resulted in two treatment means per group, one for each treatment period or one PEB mean and one NEB mean. Similarly, the average of the LSM from the two samples prior to the start of the each treatment period (day 10, 14 and 46, 50) for each group were calculated:

E: ½ ($\mu_{A,10}$ + $\mu_{A,14}$) = estimated LSM for group A, pre-treatment period 1 (pre-NEB);

F: ½ ($\mu_{A,46}$ + $\mu_{A,50}$) = estimated LSM for group A, pre-treatment period 2 (pre-PEB);

G: ½ ($\mu_{B,10}$ + $\mu_{B,14}$) = estimated LSM for group B, pre-treatment period 1 (pre-PEB); and,

H: ½ ($\mu_{B,46}$ + $\mu_{B,50}$) = estimated LSM for group B, pre-treatment period 2 (pre-NEB).

These averages were combined in a contrast that corrected the LSM of the treatment periods for pre-treatment values:

½ (A - E) + ½ (D - H) = estimate of overall NEB adjusted for pre-treatment means;

½ (C - G) + ½ (B - F) = estimate of overall PEB adjusted for pre-treatment means; and,

[½ (A - E) + ½ (D - H)] – [½ (C - G) + ½ (B - F)] = estimated overall difference between NEB and PEB adjusted for pre-treatment means.

Data are presented as LSM with 95% upper and lower confidence intervals and were backtransformed to be presented in original units.

Model 1

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \alpha\beta_{ij} + \alpha\gamma_{ik} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *k*th plate and the *l*th steer. The dependent variables monitored included:

- mean fluorescence intensity of MHC class I staining in lymphocytes, monocytes and neutrophils;
- percentage of leukocytes expressing MHC class II; and,
- mean fluorescence intensity of MHC class II staining in leukocytes.

μ = overall mean

α_i = fixed effect of group (*i* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

γ_k = fixed effect of plate (*k* = 1,2)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

$\alpha\gamma_{ik}$= interaction of *i*th group and *k*th plate

$\beta\gamma_{jk}$= interaction of *j*th day and *k*th plate

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all *i,j,k,l* and equally correlated across time within the *l*th steer, allowing for heterogeneity in variance over time (i.e., the repeated measures option of SAS PROC MIXED, Type=CSH).

Model 2

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijkl}$$

Where: Y_{ijkl}= observation of the *i*th group on the *j*th day from the *k*th steer. The dependent variables monitored included:
serum NEFA;
body weight;
calculated energy balance;
total circulating leukocytes;
circulating lymphocytes;
circulating monocytes; and,
circulating neutrophils.

μ = overall mean

α_i = fixed effect of group (*i* =A, B)

β_j = fixed effect of day (*j* =4,5....16)

$\alpha\beta_{ij}$= interaction of *i*th group and *j*th day

e_{ijkl}= error term

The error terms were assumed to be normally distributed over all *i,j,k,l* and equally correlated across time within the *k*th steer, allowing for heterogeneity in variance over time (i.e., the repeated measures option of SAS PROC MIXED, Type=CSH).

Results

A. Energy Balance

Results are as described in Chapter 2.

B. Expression of MHC Class I and Class II

There was no effect of NEB on the expression of MHC class I (Table 4.1). There also was no effect of NEB on the percentage of cells expressing MHC class II (Table 4.2). However, there was a small but significant increase in the MFI of MHC class II (P = 0.03; Figure 4.3). However, the difference was only significant during the second treatment period.

Discussion

Protection against infection depends first on physical barriers and then on non-specific or innate mechanisms which occur within minutes to hours. Finally, protection depends on the specific or adaptive immune response, which provides antibody and T-lymphocyte responses. These require a week or more to be fully expressed but have "memory" and are long-lasting (Ojcius et al., 1994). During the cell-mediated response, the T-cell receptor (TCR) recognizes antigen as a peptide fragment bound to MHC. T-lymphocytes are activated by specific binding to peptide-MHC complexes on the surface of antigen-presenting cells such as macrophages.

There are two classes of MHC. Class I molecules are expressed constitutively by all nucleated cells, including leukocytes (Figure 4.1a) and bind to peptides derived from endogenously synthesized molecules such as viral peptides. These are recognized by the cytotoxic or CD_8 +T lymphocytes (Ojcius et al., 1994). In the present study all leukocytes expressed MHC I and this expression was not affected by NEB.

Specialized antigen-presenting cells (APC) such as monocytes and B-cells also express MHC class II molecules (Blanden, 1986). This is consistent with the current study because a small population of bovine leukocytes stained positively for MHC class II (Figure 4.1b). Antigens presented in MHC class II molecules are recognized by T-helper or CD4+ T lymphocytes (Blanden, 1986) which can then activate phagocytes of the innate immune system as well as activate the B-lymphocytes of the adaptive immune system (Figure 1.3). T-

lymphocytes require that antigen be bound to MHC before it can be recognized (Springer, 1990). Thus, MHC can influence susceptibility to infectious diseases via MHC-restricted CD8+ and CD4+ T-cell responses (Blanden, 1986). The percentage of MHC class II positive cells was not affected by NEB in the present study but in the second treatment period NEB increased the expression of MHC class II per cell.

A study in pigs found that rotavirus enhanced expression of MHC class I and class II mRNA in the jejunum of the small intestine. Malnutrition further enhanced MHC class I mRNA expression but suppressed MHC class II mRNA expression and resulted in a greater duration of infection (Zijlstra et al., 1999). A second study in pigs conducted during the period of weaning found that jejunal MHC class I mRNA expression was lower on day 1 postweaning compared with day 0 and day 4. Jejunal MHC class II mRNA expression also decreased but not to a level of significance. The observed decreases were attributed to inadequate feed intake during the transition from milk to solid feed (McCracken et al., 1999). These studies suggest that malnutrition can alter MHC expression and potentially increase susceptibility to and duration of infections because MHC is required for antigen presentation to T-lymphocytes..

In cattle, the number of MHC class II expressing leukocytes is decreased after parturition (Van Kampen and Mallard, 1997). This may be due to multiple changes associated with parturition including NEB. However, our results show virtually no change in MHC class II expression due to NEB and no effect on MHC class I expression. This is quite the opposite of what was observed in pigs.

However, the pig studies included either an immune challenge (rotavirus infection; Zijlstra et al., 1999) or the stress of weaning. Also, cortisol is elevated around parturition in cattle and is known to have immunosuppressive effects (Preisler et al., 1999). The steers used in the present study did not undergo any experimentally induced infection and were not clinically ill during the trial. There was no stress imposed other than the stress of NEB. The results of the present study suggest that NEB alone in cattle does not alter MHC expression. Future studies should examine the expression of MHC class I and class II in periparturient dairy cows as well as monitor the influence of cortisol on expression of these molecules during NEB and PEB.

Chapter Four

Tables and Figures

Table 4.1. Least squares means of MHC class I mean fluorescence intensity (MFI) for bovine blood leukocytes from steers in positive (PEB) or negative energy balance (NEB).

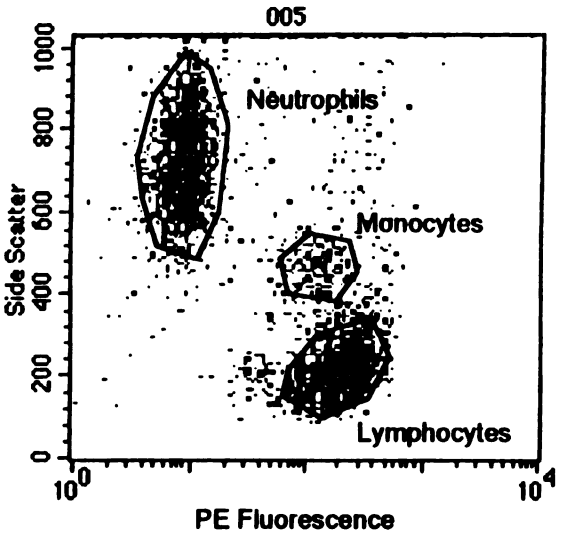
	PEB (95% Confidence Limit)	***NEB*** (95% Confidence Limit)	***P-value***
Lymphocyte MFI	83.1 (61.6, 111.1)	89.1 (66.7, 117.9)	.5
Monocyte MFI	68.0 (43.8, 105.6)	73.0 (47.5, 113.3)	.5
Neutrophil MFI	8.1 (6.6, 9,9)	7.2 (6.6, 9.8)	.3

Table 4.2. Least square means of MHC class II expression, measured as percentage of positively stained cells and mean fluorescence intensity (MFI), for bovine blood leukocytes from steers in positive (PEB) and negative energy balance (NEB).

	PEB (95% Confidence Limit)	***NEB*** (95% Confidence Limit)	***P-value***
% of Positive Leukocytes	19.3 (11.5, 28.6)	20.2 (12.2, 29.5)	.4
Leukocyte MFI Treatment Period 1	78.3 (54.6, 121.5)	94.6 (66.7, 134.3)	.7
Leukocyte MFI Treatment Period 2	330.3 (221.4, 492.7)	298.9 (181.3, 492.7)	.003

Figure 4.1. Flow cytometric identification for a representative animal of A)MHC class I, and B)MHC class II differentiated bovine leukocyte populations based on PE fluorescence (log scale; x-axis) and side scatter characteristics (granularity; y-axis). In A) three populations were identified, neutrophils, monocytes and lymphocytes. In B) two populations were identified, MHC class II positive leukocytes and MHC class II negative leukocytes.

A. MHC class I



A)MHC
based on
nularity;
and
ve

B. MHC class II

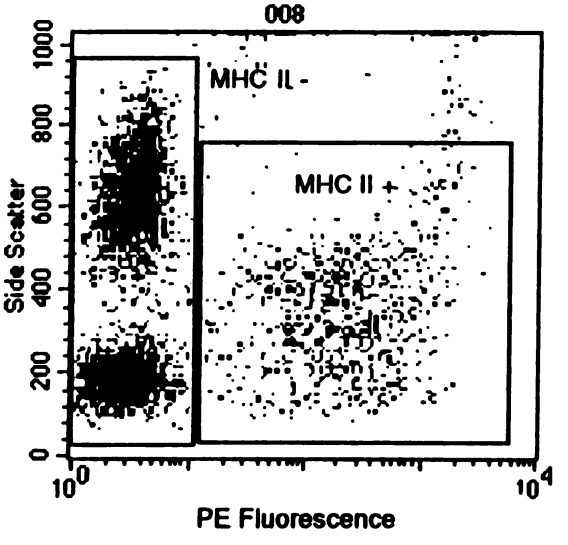
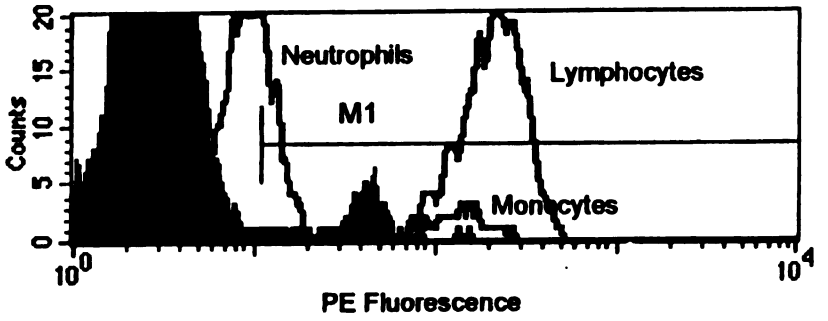


Figure 4.2. Differential expression of A)MHC class I, and B)MHC class II in PE stained bovine leukocytes was determined by PE-mean fluorescence intensity (log scale; x-axis). Shown in the histograms are representative peaks for each leukocyte type from a representative animal. In A) the solid green peak represents the negative control, the red peak represents neutrophils, the blue peak represents lymphocytes, and the purple peak represents monocytes. In B) the solid green peak represents the negative control, the red peak represents leukocytes that did not stain positively for MHC class II, and the purple peak represents leukocytes that stained positively for MHC class II.

A. MHC class I



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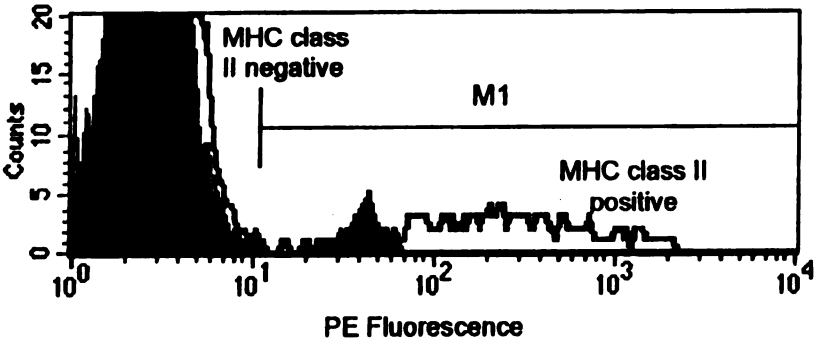
ple peak

B. MHC class II



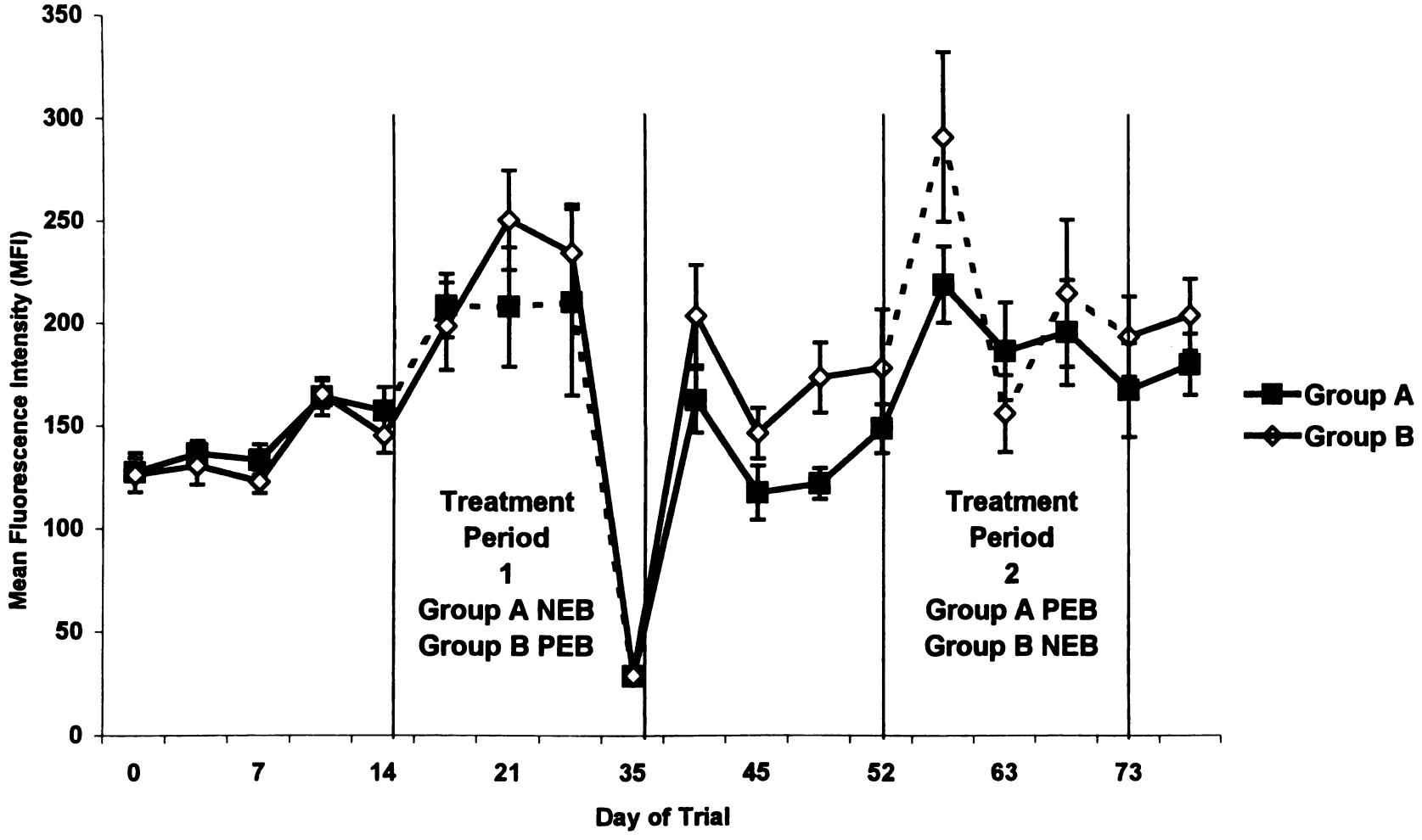


Figure 4.3. Mean fluorescence intensity of MHC class II expression by blood leukocytes from steers in positive (PEB) and negative energy balance (NEB). Each line (squares or diamonds) follows one group through the entire trial. Lines are dashed when steers of the groups are fed NEB, and solid when the group was fed PEB.

Chapter 5

GENERAL DISCUSSION AND IMPLICATIONS

The dairy cow is immunosuppressed around the time of parturition (Table 1.2). At this time, dairy cows are more susceptible to multiple metabolic and infectious diseases, including mastitis (Oliver and Mitchell, 1983). There are many physiologic and metabolic changes associated with parturition including decreased dry matter intake and increased demand for energy for expulsion of the calf and onset of lactation (Grummer, 1995). This causes the cow to be in NEB and results in mobilization of body energy reserves in the form of serum NEFA. A study on commercial dairy farms found that cows with high NEFA prepartum had an increased incidence of mastitis and retained placenta after calving (Dyk et al., 1995). High liver fat in dairy cows is associated with a reduction in the number of peripheral leukocytes (Reid et al., 1983), and the mitogenic response of bovine lymphocytes is attenuated when these cells are preincubated with β-hydroxybutyrate or acetoacetate, which are found in the circulation of cattle in severe NEB (Targowski and Klucinski, 1983). Protein energy malnutrition (PEM) in children, a large component of which is NEB, is associated with significant impairment of cell-mediated immunity, phagocyte function, complement concentration and activity, secretory immunoglobulin A antibody concentrations, and cytokine production (Chandra, 1997). Therefore, I postulated that NEB was a key component of reduced immunocompetence and increased disease susceptibility in dairy cattle.

One possible mechanism by which NEB could affect immune function is via metabolic hormones such as GH and IGF-I. NEB uncouples the GH/IGF-I axis in cattle. Circulating concentrations of IGF-I decrease significantly during NEB even though GH concentrations may be high (Yung et al., 1996; McGuire et al., 1992a,b; Clemmons and Underwood, 1991; VandeHaar et al., 1995; Sharma et al., 1994). Uncoupling of the GH/IGF-I axis during NEB is due partly to down-regulation of GH receptors in the liver (Yung et al., 1996; McCusker, 1998). In a well-fed dairy cow, GH and IGF-I positively affect immune function (Burton et al., 1994). If NEB also alters expression of GH and (or) IGF-I receptors on immune cells, the normal immunoenhancing actions of these hormones on the immune system could be impaired, resulting in decreased immunocompetence.

Evidence for the importance of these hormones is shown in cultured human lymphoid cells, untransformed lymphocytes from peripheral blood, splenocytes, polymorphonuclear and mononuclear leukocytes, and tissue macrophages from several species, including cattle (reviewed by Burton et al., 1994). All of these cell types bind and respond to GH and IGF-I via specific receptors. Immune cells also produce and secrete both GH and IGF-I and these hormones can mediate immune cell function. Receptors for GH and IGF-I are down-regulated with increasing local concentrations of these hormones (Lesniak and Roth, 1976; Lesniak et al., 1974; Stuart et al., 1991; Zhao et al., 1992). This suggests the possibility that local and (or) circulating GH and IGF-I regulate immune function. If NEB uncouples the GH/IGF-I axis at the liver via down-regulation of receptors, we postulated that the same could be true in the immune

system and result in decreased immunocompetence. We monitored expression of GH and IGF-I receptors, as well as key adhesion molecules and MHC molecules on circulating leukocytes to test this possibility.

Given the information presented above, the overall hypothesis of this research was that NEB alters the expression of GH and (or) IGF-I receptors on bovine blood leukocytes (Figure 2.1; Chapter 2). The secondary hypothesis was that NEB also alters gene expression of key adhesion (CD62L, CD18, CD11b) and antigen presenting molecules (MHC class I, MHC class II) by bovine blood leukocytes. The implications are that this would cause decreased immunocompetence and increased disease susceptibility in cattle during NEB. To begin to test this hypothesis, we developed a steer model of bovine periparturient NEB that would both uncouple the GH/IGF-I axis and eliminate possible confounding effects of reproductive and other metabolic hormones, such as progesterone, estrogen and glucocorticoids, which fluctuate greatly around parturition. We developed immunostaining and fluorescence-activated flow cytometric protocols to monitor hormone receptors, adhesion molecules and antigen-presenting molecules on bovine blood leukocytes. We used these assays to monitor expression of these important receptors and molecules during severe NEB and compared results with those of animals in PEB.

As expected from observations on periparturient cows, our model of NEB decreased body weight, increased serum NEFA, and decreased serum IGF-I thereby confirming that NEB was imposed by our nutritional regimen. However, the GH results were difficult to interpret. NEB slightly decreased serum GH pre-

feeding, but increased serum GH at 3 h post-feeding. A previous study using a similar model of NEB in cattle showed that NEB increased the mean serum GH concentration over a 10-h period with the greatest increase occuring 2 h post-feeding (VandeHaar et al., 1995). Thus, our results suggest that the GH/IGF-I axis was uncoupled at the liver during NEB in this study and in the immune system as well (Yung et al., 1996; McGuire et al., 1992a,b; VandeHaar et al, 1995; Pell et al., 1993; Elsasser et al., 1989; Bass et al., 1990; Breier, et al., 1989; Clemmons and Underwood, 1991).

NEB increased the number of circulating lymphocytes and also decreased the percentage of lymphocytes expressing GH receptor from 50 to 42% and the percentage of monocytes expressing IGF-I receptor from 90 to 88%. This slight decrease, although significant, may not have any biological importance.

There are two possible explanations for the decreased percentage of lymphocytes expressing GH receptor. A specific subpopulation of lymphocytes may have down-regulated GH receptor in response to NEB or, perhaps a subpopulation of lymphocytes left the circulation in response to NEB. There are many subpopulations of lymphocytes and it is feasible that a population down-regulated the GH receptor due to internalization of the bound hormone (Allevato et al., 1995). Addition of GH to cultured human lymphoid IM-9 cells causes down-regulation of the GH receptor (Lesniak and Roth, 1976). Once GH binds to the GH receptor, it is internalized, suggesting that the duration and degree of sensitivity to GH may be regulated at the cellular level (Lesniak and Roth, 1976).

If a population of lymphocytes internalized their GH receptors due to excessive GH binding, it would account for the observed decrease.

A study using bulls found that B-lymphocytes made up about 10% of the circulating population of leukocytes (Burton and Kehrli, 1995). Because approximately 10% fewer circulating lymphocytes expressed GH receptor, B-lymphocytes could be the affected population.

It is also possible that the decreased proportion of lymphocytes expressing GH receptor during NEB may be the result of a population of lymphocytes leaving the peripheral blood. This is consistent with a study in which exogenous GH reduced the number of lymphocytes in the peripheral blood of cows (Burton et al., 1992). In the present study, a loss of lymphocytes from the total population that exhibited low intensity staining (i.e., less GH receptor per cell) might account for the small increase in MFI of the remaining cells that expressed GH receptor. However, this scenario is unlikely because NEB increased the number of circulating lymphocytes by 17%.

The effects of GH on immune cells may be mediated by IGF-I, thus requiring the presence of IGF-I receptors. In the present study, NEB decreased the percentage of monocytes that expressed IGF-I receptor by 2%. Although statistical analysis indicated significance, a 2% change in monocytes expressing IGF-I receptor may not be biologically significant in decreasing GH or IGF-I action on monocytes. However, it could be possible that this, combined with a decrease in GH binding to immune cells could decrease immunocompetence.

One mechanism by which the decrease in the percentage of lymphocytes expressing GH receptors may decrease immunocompetence is by impairing blastogenesis. Both GH and IGF-I normally enhance lymphocyte blastogenesis (Burton et al., 1991; Zhao et al., 1992). Decreased binding of GH may result in defects in lymphocyte function. Normally, mitogen-stimulated lymphocytes produce lymphokines, such as gamma-interferon, that are known to activate phagocytes (Burdach et al., 1987). Also, antibody secreted by lymphocytes facilitates phagocytosis (Kehrli et al., 1989a). Therefore, if lymphocyte blastogenesis is decreased, neutrophil function also is adversely affected. Kehrli et al. (1989a) found significant correlation between alterations in lymphocyte blastogenesis and alterations in neutrophil function in periparturient dairy cows that were most likely undergoing NEB. Kehrli et al. (1989a) also observed an association of intramammary infection with suppressed lymphocyte blastogenesis at this time, suggesting that decreased lymphocyte blastogenesis can decrease immunocompetence and increase susceptibility to infectious disease.

To further examine the effect of NEB and separation of the GH/IGF-I axis on immunocompetence, expression of key adhesion (CD62L, CD11b, and CD18) and antigen-presenting (MHC class I and MHC class II) molecules by bovine blood leukocytes also was monitored. NEB increased the expression of CD62L by neutrophils and slightly increased MHC class II expressed by mononuclear leukocytes.

These results are quite different from those observed during periparturient immunosuppression. Recently, Lee and Kehrli (1998) found that neutrophil expression of CD62L is decreased following parturition. In addition, MHC class II expression remains relatively constant until calving and then decreases after calving (Van Kampen and Mallard, 1997).

Malnutrition suppresses MHC class II expression in pigs infected with rotavirus (Zijlstra et al., 1999). In the present study, NEB did not substantially alter MHC class II expression, suggesting that NEB without an immune challenge is not sufficient to suppress MHC class II expression.

This is the first study to demonstrate that NEB can decrease the percentage of bovine mononuclear leukocytes that express receptors for GH and IGF-I. However, decreased expression of these receptors did not seem to adversely affect expression of key adhesion (CD62L, CD11b, and CD18) or antigen-presenting (MHC class I and MHC class II) molecules. In fact, expression of CD62L and MHC class II actually was enhanced during NEB in this study.

However, this study examined only the effect of insufficient energy and protein on immunocompetence. Although the PEB diet contained greater amounts of some vitamins and minerals, both diets met or exceeded NRC (1989) requirements for all vitamins and minerals. During the periparturient period there is a decrease in mineral and vitamin intake below requirements because of the overall decrease in dry matter intake. Perhaps other nutritional deficiencies may adversely effect the immune system. For example, a study where periparturient

dairy cows were supplemented with Vitamin E found that neutrophils from control cows produced two-fold less superoxide anion after parturition than the supplemented cows (Politis et al., 1995). This indicates that neutrophils from the control cows were less able to kill bacteria than those supplemented with Vitamin E and that immune function is enhanced by supplementing only one aspect of the diet.

The findings of the present study using a steer model suggest that the immunosuppression associated with the periparturient period may not be due to NEB alone, but may be influenced by one or a combination of several other endocrine changes or dietary factors that occur at this time. At the time of parturition there are a myriad of changes including a sudden decrease in progesterone, insulin, and IGF-I, and increases in cortisol, estrogen, and GH. Perhaps increased serum cortisol concentrations may impair neutrophil trafficking via activation of glucocorticoid receptors (Preisler et al., 1999). Also, binding of other hormones such as progesterone, estrogen and even prolactin to their receptors affects gene expression in the immune system. For example, administration of progesterone to steers increased random migration of neutrophils and impaired the ability of neutrophils to kill bacteria (Roth et al., 1982).

The use of steers in this study was intended to prevent the interaction of NEB with these endocrine changes with the exception of GH and IGF-I. However, in so doing, the right combination of endocrine changes needed for

decreased immunocompetence may not have been included. Therefore, this study may need to be done in periparturient dairy cows.

This study also only examined basal expression of these indicators of immunocompetence. Perhaps NEB only suppresses the immune system when an animal faces an immune challenge (e.g., infection). A similar model of bovine NEB could be used in combination with an immune challenge to determine the ability of the immune system to regulate expression of these adhesion and antigen-presenting molecules during NEB.

Finally, another possibility is that NEB only has negligible effects on immunocompetence compared with other factors, at least in periparturient dairy cows. Regardless, this study clearly demonstrates that severe NEB, per se, uncoupled the GH/IGF-I axis in steers and decreased the percentage of mononuclear leukocytes expressing receptors for GH and IGF-I. In addition, expression of CD62L and MHC class II was increased during NEB. Therefore, NEB did not jeopardize immunocompetence, at least as indicated by basal expression of adhesion and antigen-presenting molecules by circulating leukocytes.

Chapter 6

RECOMMENDATIONS FOR FUTURE RESEARCH

As mentioned in previous chapters, NEB decreased the percentage of resting bovine mononuclear leukocytes expressing GH and IGF-I receptors. This, however, did not adversely affect expression of key adhesion (CD62L, CD11b, and CD18) or antigen-presenting (MHC class I and MHC class II) molecules. However, the steers in the present study did not undergo any immune challenge and bovine leukocytes were not stimulated before assay. Much of the literature reports alterations in leukocyte function after activation. This study simply measured expression of these receptors and molecules on resting leukocytes collected from the circulation.

Many of the immunoenhancing effects of GH and IGF-I are not observed until leukocytes are activated by a mitogen or pathogen. The present study examined the effect of NEB on resting leukocytes and may not provide the whole picture. A study should be conducted where leukocytes from dairy cows in NEB are activated with a mitogen *in vitro* to determine the ability of these cells to regulate expression of adhesion and antigen-presenting molecules in response to mitogenic stimulation. Another way to examine the effect of energy balance on immunocompetence would be to give an immune challenge to a cow in NEB and PEB and monitor leukocyte expression of adhesion and antigen-presenting molecules *in vivo.* Such a study is being developed in our laboratory. Early lactation primiparous dairy cows will be fed for either NEB or PEB and will be

given an intramammary infusion of lipopolysaccharide. This will cause an inflammatory response, enabling us to measure the ability of leukocytes to migrate into the mammary gland. Leukocytes will also be harvested for *in vitro* activation. The results of this study will provide conclusive evidence as to whether or not NEB alters immunocompetence.

Alternatively, perhaps the cause of periparturient immunosuppression is an interaction of elevated NEFA or ketones, such as acetoacetate and β-hydroxybutyrate, with changes in other hormones such as insulin, progesterone and cortisol. One way to investigate this is to perform a study similar to the present study but to use periparturient cows. This would incorporate all the changes associated with parturition. Currently, there is no information on bovine leukocyte expression of receptors for GH and IGF-I during the periparturient period. The proposed study would provide this information as well as data on other immunocompetence indicators (i.e. adhesion molecules) that could be correlated to changes in hormone receptors and changes in NEFA concentration.

To further investigate the role of malnutrition on immune function, studies could be designed that feed diets deficient in only specific nutrients. This would determine exactly what feed component is most important to the periparturient period.

Clearly, the results of the current study do not implicate NEB as primarily responsible for periparturient immunosuppression. However, the studies mentioned above may help elucidate exactly what role NEB does play, if any. This information has the potential to improve not only dairy cow health via

nutrition, but also human health. Traditionally, studies in human malnutrition have been mostly anecdotal. The use of dairy cattle as a model of NEB provides a controlled environment that is unattainable in human research and may provide important data for exploring the mechanisms by which malnourished individuals become immunosuppressed and highly susceptible to infectious disease.

APPENDIX

APPENDIX

ANTIBODY SPECIFICITIES AND THEIR SOURCES

Specificity	Antibody	Isotype	Company	Dilution
Anti-IGF-I R	1H7	IgG1	Pharmingen	1:400
Anti-GH R[1]	R1-M1-B11	IgG2a	Monsanto Inc.	1:10
Anti-MHC I	H58A	IgG2a	VMRD[2]	3.5 µl/ml
Anti-MHC II	CAT82A	IgG1	VMRD[2]	7.0 µl/ml
Anti-CD62L	BAQ92A	IgG1	VMRD[2]	7.0 µl/ml
Anti-CD18	BAQ30A	IgG1	VMRD[2]	7.0 µl/ml
Anti-CD11b	MM12A	IgG1	VMRD[2]	7.0 µl/ml
Anti-mouse IgG1	(FITC)[3]	Secondary	Caltag	1:10
Anti-mouse IgG1	(PE)[4]	Secondary	Caltag	1:50
Anti-rat IgG2a	(FITC)[3]	Secondary	Pharmingen	1:10
Anti-mouse IgG2a	(PE)[4]	Secondary	Caltag	1:100
Negative FITC control		IgG1	Dako	7.0 µl/ml
Negative PE control		IgG1	Dako	7.0 µl/ml

[1]Generously donated by Dr. Wesley Warren (Protiva Unit, Monsanto Inc., St. Louis, MO).
[2]Veterinary Medical Research and Development
[3]FITC = Fluorescein Isothiocyanate
[4]PE = Phycoerythrin

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