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thesis entitled

GLUCOCORTICOID RECEPTOR EXPRESSION IN BOVINE BLOOD LEUKOCYTES AND THE EFFECTS OF PARTURITION

presented by

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has been accepted towards fulfillment
of the requirements for

MS degree in Animal Science

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Date 13 July 1999

GLUCOCORTICOID RECEPTOR EXPRESSION IN BOVINE BLOOD LEUKOCYTES AND THE EFFECTS OF PARTURITION

By

Mara Therese Preisler

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Animal Science

1999

ABSTRACT

GLUCOCORTICOID RECEPTOR EXPRESSION IN BOVINE BLOOD LEUKOCYTES AND THE EFFECTS OF PARTURITION

By

Mara Therese Preisler

During the periparturient period there is an increase in blood concentrations of the stress hormone, cortisol, which carries out its function of maintaining whole-body homeostasis by binding to cytoplasmic glucocorticoid receptors (GR) in target cells. The objectives of this study were to monitor levels of bovine leukocyte GR expression throughout parturition and to determine if there were associations between GR expression, cortisol, and leukocyte counts. To accomplish these objectives, a two-color fluorescent staining and flow cytometric assay was developed to monitor cytoplasmic GR. Leukocyte sub-populations were differentiated by degree of CD45 expression (phycoerythrin-stained). Glucocorticoid receptor expression was detected by the intensity of fluorescein isothiocyanate- conjugated dexamethasone (a GR ligand). Flow cytometric analysis of dual-labeled cells revealed that monocytes had the highest GR expression followed by neutrophils and then by lymphocytes. Then, using the developed assay, GR expression in the specific leukocytes was monitored throughout parturition. Blood from 13 test animals was sampled on -28 d, -21 d, -14 d, -7 d, 0 h, 12 h, 24 h, 36 h, 48 h, 7 d, 14 d relative to parturition. Samples from 10 mid-gestation cows were used as controls. During parturition there was significant down-regulation of leukocyte GR expression (39% in neutrophils, 42% in lymphocytes, and 47% in monocytes; $P < 0.01$) relative to leukocytes from control animals. There was also a 7-fold increase in blood cortisol concentrations at calving ($P < 0.001$). Correlations between cortisol and GR expression were negative ($P < 0.05$) for all leukocyte types. Cortisol and GR expression correlated with leukocytosis ($P < 0.001$). These observations provide preliminary evidence that cortisol-activated GR may be involved in altered leukocyte phenotypes and functions of periparturient dairy cows.

To Doug – for his patience, support, and love

that kept me "mooooving" in the right direction.

ACKNOWLEDGEMENTS

I would like to thank many people because so many of you have made the past three years a wonderful experience. First, I would like to acknowledge the MSU dairy farm crew. They were instrumental in paging me whenever I was needed and keeping me laughing when I was beyond overtired. Thanks to Yan Yuan for assisting me with the cortisol assays. Next, I would like to acknowledge everyone that has been a part of the "Estrogen Lab" for making the last three years so special. I really appreciated all the help and support when times were hectic, especially during the trial. Thanks for making life in the basement so enjoyable.

I would like to thank my committee members, Dr. Jeanne Burton (advisor), Dr. Patty Weber, Dr. Rob Tempelman, Dr. Henry Hunt, and Dr. Ron Erskine. Working with all of you has been "udderly" delightful. Thank you for all your enthusiasm, ideas and support. In particular, thank you for all your critical thinking when it came to manuscript and defense time.

Jennifer Wells, what can I say – you have been a great colleague and very special friend. Thanks for always sharing your entertaining Calvin and Hobbes cartoons and eating ice cream with me every chance we could (Mint Chip Rules!!).

A special thanks to Patty Weber. You are a great "Lab Mama". Thank you for being an outstanding committee member, teacher, and friend. I will dearly miss our heart to hearts in the lab and at the wee hours of the morning and singing La Coo Coo Ra Cha in delerium.

Jeanne, thanks for opening up the world of science to me. You do not know how much you have helped me develop as a scientist and as a person. Your guidance and friendship are beyond words. I will miss your passion for science, our great bonding

sessions, going to meetings with you, talking flowers, goofing around in the lab, and your artwork with red pens.

Finally, I would like to thank the rest of my support staff. Thanks to Rachel and Andy Mitchell for being wonderful friends and letting me crash with them while I finished this thesis. Thanks to Doug for being there every step of the way and making even the hard times seem fun. Thanks for everything, babe, you are the best. And, last but not least, I would like to acknowledge and thank my parents, Steve and Diane, and sister, Liz, for always being there and supporting me, even if it meant Christmas with the cows. Thanks for your neverending love, encouragement, and support. You guys mean the world to me.

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LIST OF ABBREVIATIONS

ACTH = Adrenocorticotrophic Hormone

AVP = Arginine Vasopressin

CD18 = Cluster of Differentiation 18

CD45 = Cluster of Differentiation 45

CON A = Conconavalin A

CRH = Corticotropin-Releasing Hormone

DMSO = Dimethyl Sulfoxide

DEX = Dexamethasone

ETOH = Ethanol

FITC = Fluorescein Isothiocyanate

FSC = Forward Scatter

GC = Glucocorticoids

GR = Glucocorticoid Receptors

GRE = Glucocorticoid Response Elements

HPA = Hypothalamic-Pituitary-Adrenal Axis

Hsp90 = Heat Shock Protein 90

LSM = Least Square Means

MFI = Mean Fluorescence Intensity

PBS = Phosphate Buffered Saline

PE = Phycoerythrin

PHA = Phytohemagluttinin

RU486 = Mifepristone

SSC = Side Scatter

FOREWARD

Chapters Three and Four of this thesis are manuscripts that have been accepted for publication in the *American Journal of Veterinary Research* (Chapter Three) and *Journal of Dairy Science* (Chapter Four). Appendix Two contains the letters granting permission that these manuscripts can be used in this thesis. The titles and authors are listed below:

Preisler, M. T., P. S. D. Weber, R. J. Tempelman, R. J. Erskine, H. Hunt, J. L. Burton. 1999. Glucocorticoid Receptor Down-Regulation in Neutrophils of Periparturient Cows. – Accepted by the American Journal of Veterinary Research. In Press.

Preisler, M. T., P. S. D. Weber, R. J. Tempelman, R. J. Erskine, H. Hunt, J. L. Burton. 1999. Glucocorticoid Receptor Expression Profiles in Mononuclear Leukocyte Populations of Periparturient Dairy Cattle. – Accepted by the Journal of Dairy Science. In Press.

INTRODUCTION

As dairy cows enter the periparturient period of their production cycle, they experience physical, nutritional, and metabolic changes that may be perceived as stress. Parturition itself is associated with elevated blood concentrations of the stress hormone, cortisol, in the maternal circulation (Goff et al., 1989; Guidry et al., 1976). On one hand, cortisol is crucial for the expulsion of the fetus and onset of lactation. On the other hand, however, cortisol causes dysfunctions of blood leukocytes that may contribute to increased susceptibility to infectious diseases in early lactation (Burton and Kehrli, 1996; Burton et al., 1995; Lee and Kehrli, 1998; Kehrli et al., 1998).

Cortisol, a natural glucocorticoid (GC) hormone, carries out its functions by binding to cytoplasmic glucocorticoid receptors (GR) in target cells. Once bound to GR, the GC-GR complex is activated and translocates into the nucleus to bind to regulatory regions of glucocorticoid responsive target genes (Norman and Litwack, 1997; Oakley and Cidlowski, 1993). GR then becomes a ligand-activated transcriptional regulator of the target genes, either enhancing or repressing their expression, depending on the gene. Therefore, GC activation of GR is a major mechanism used by cells to alter their phenotype and function to cope with and adapt to stress.

Dexamethasone (DEX) is a synthetic GC that binds with higher affinity to GR than cortisol. This makes DEX a useful molecular tool to study the effects of GCs on leukocyte phenotypes and functions. Previous research has demonstrated that leukocyte phenotypes and functions are dramatically altered in cattle challenged with DEX (Burton and Kehrli, 1995; Burton and Kehrli, 1996; Burton et al., 1995; Lohuis et al., 1988;

Nonecke et al., 1997; Roth and Kaeberle, 1981; Roth et al., 1982; Wesley et al., 1989), implicating involvement of GR activation in generalized immune suppression and disease susceptibility. When DEX was administered to lactating cows with subclinical intramammary infections, increased bacterial shedding in milk was observed that often lead to the development of clinical mastitis (Burton and Kehrli, 1995; Lohuis, et al., 1988; Wesley et al., 1989). Burton et al. (1995) and Burton and Kehrli (1995) elucidated one molecular mechanism for GC-induced mastitis susceptibility by demonstrating that important adhesion molecules (CD62L and CD18) were down-regulated on the plasma membranes of neutrophils in DEX-treated cows. This leads to dysfunctional trafficking and migration functions of the neutrophils such that the leukocytes were not able to migrate from blood into infected mammary parenchyma. On the other hand, DEX was relatively ineffective in altering mononuclear leukocyte expression of CD62L and CD18, but instead inhibited the capacity of these leukocytes to produce cytokines and antibodies *in vitro* (Burton and Kehrli, 1996). The implications from this research are that leukocytes express GR and that GCs activate GR in leukocytes to cause altered gene expression related to generalized immune suppression.

The DEX model of bovine immune suppression has recently been extended to a natural model of GC-induced immunosuppression in dairy cows, namely parturition. Lee and Kehrli (1998) demonstrated that neutrophils of periparturient dairy cows also down-regulate CD62L and CD18 in conjunction with increased concentrations of cortisol in maternal blood. As in the DEX model, CD62L and CD18 down-regulation was associated with neutrophilia and increased mastitis susceptibility, again implicating GC-activated GR as a possible mechanism for generalized immunosuppression.

In studies using human cells, cell lines, and GR gene expression systems, the degree of transcriptional regulation by GR is dependent on the level of GR expressed in the cytoplasm of the cells (Bamberger et al., 1996; De Rijk and Berkenbosch, 1994; Dong et al., 1988). Furthermore, these cells and cell systems have been used to show that GR is autoregulatory: when GC concentrations are increased, homologous down-regulation of GR occurs (Burnstein and Cidlowski, 1992; Oakley and Cidlowski, 1993). Homologous down-regulation of GR has been clearly demonstrated in human lymphocytes (Schlecte et al., 1982; Shipman et al., 1983). To our knowledge, however, no studies have demonstrated the presence or regulation of GR in response to glucocorticoids in neutrophils, nor in any leukocyte of periparturient dairy cows. Therefore, the first objective of the current study was to determine whether bovine leukocytes express GR, and if so, was there differential expression of GR in different leukocyte types (Chapter Two). Next, an experiment was designed to test the effects of parturition on neutrophil GR expression (Chapter Three), and monocyte and lymphocyte GR expression (both in Chapter Four). The final objectives were to determine if GR expression was correlated with blood cortisol concentration in periparturient dairy cows, and whether blood cortisol and (or) leukocyte GR expression were correlated with circulating numbers of each leukocyte type (Chapters Three and Four).

Results of these studies are important because they could implicate GR as a mediator of GC-induced immune suppression, especially in periparturient cows. If true, bovine leukocyte GR could become a key target for the design and development of future pharmaceuticals intended to prevent or treat infectious diseases in periparturient and otherwise stressed dairy cows.

CHAPTER ONE

Literature Review

I. GLUCOCORTICOIDS AND THE GLUCOCORTICOID RECEPTOR

A. Endogenous Glucocorticoids

Glucocorticoids (GC) are steroid hormones that are vital in maintaining homeostasis. Glucocorticoids are involved in glycogen and lipid metabolism, immune function, cardiovascular function, and behavorial responses to stress (Chrousos, 1995; Norman and Litwack, 1997). Cortisol (corticosterone in rodents) is the major endogenous glucocorticoid, and is synthesized and released by the adrenal gland following activation of the hypothalamic-pituitary-adrenal- (HPA) axis in response to stressors. When the body responds to a stressor, the brain receives the message in the hypothalamus and stimulates an increase in cortisol secretion (Figure 1.1).

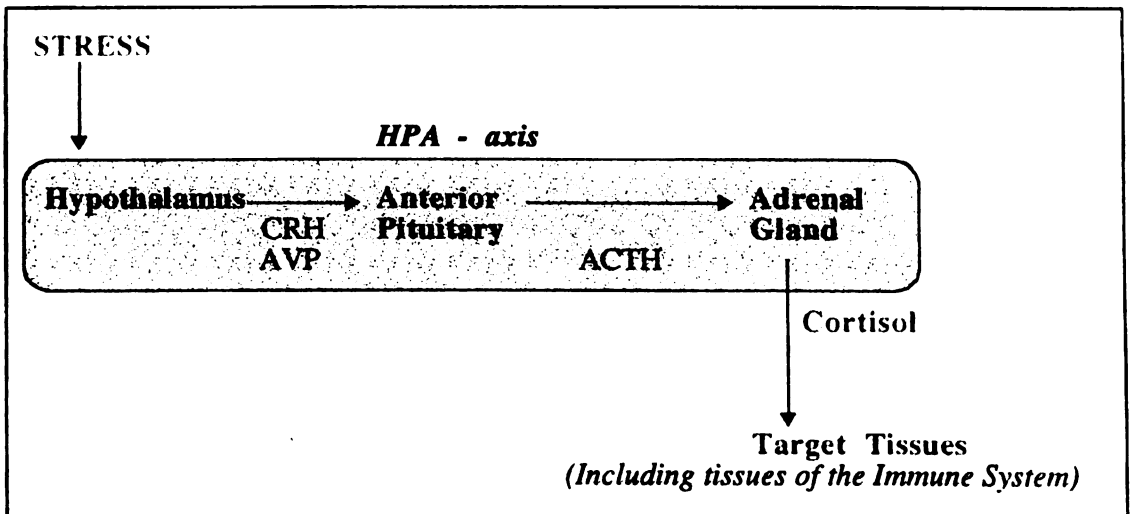


Figure 1.1 - Activation of the HPA-axis leads to cortisol secretion from the adrenal cortex.

In the hypothalamus, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are secreted to permit the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. Then, ACTH stimulates the adrenal gland to release cortisol. Subsequently, cortisol is secreted into the circulation where it is transported to target cells to carry out its many homeostasis-regulating functions by binding to glucocorticoid receptors (GR) in the cytoplasm of these cells. Long- and short-loop negative feedback mechanisms of cortisol, ACTH, CRH, AVP, and GR keep the HPA-axis and cellular sensitivity to GCs in equilibrium to maintain overall homeostasis.

B. Glucocorticoid Receptors

Glucocorticoids exert their hormonal actions by binding to GR that are located in the cytoplasm of GC-responsive cells, including leukocytes. The bovine GR gene has yet to be sequenced, so presented information on GR regulation and expression is drawn from what is known about this receptor molecule in other species. The human GR gene is composed of 9 exons with intervening introns; alternative splicing of exon 9 during transcription of the human GR gene yields two isoforms called α and β (Bamberger et al., 1996; Hollenberg et al, 1985; Oakley et al, 1996). GRα and GRβ differ only at their carboxy termini; GRα contains amino acids that bind with GC, while the truncated GRβ does not. Therefore, it is widely believed that GRα is the biologically relevant isoform in GC-sensitive cells.

Human GRα is a cytoplasmic protein that is approximatley 94-kDa and is composed of 777 amino acids. It belongs to the steroid hormone receptor superfamily of ligand-activated transcription regulators (Bamberger et al, 1996; Carson-Jurica et al, 1990). Consistent within this family, GR is composed of three major functional domains. The N-(amino-) terminal domain is highly variable among members of the superfamily and contains a first transactivation domain, which is directly involved in transcriptional regulation of target genes by GR. The central DNA-binding region is highly conserved across members of the superfamily and is responsible for GR binding with specific DNA

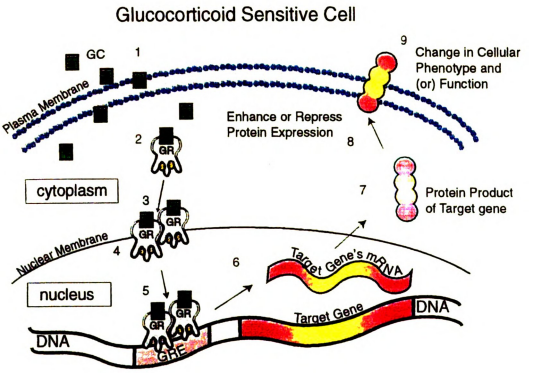


Figure 1.2 – The glucocorticoid receptor (GR) is a ligand-activated transcription regulator that alters the phenotype and (or) function of glucocorticoid (GC) sensitive cells by enhancing or repressing transcription of target genes.

sequences in the target genes, called glucocorticoid response elements (GRE). The carboxy-terminal region contains the GC-binding domain as well as sequences required for interactions of GR with heat shock protein 90 (Hsp90) and other proteins involved in nuclear translocation and GR dimerization. This domain also contains the second transactivation domain of GR (Oakley and Cidlowski, 1993; Oakley et al., 1996).

The mechanism of ligand-activated transcriptional regulation by GR is depicted in Figure 1.2. Glucocorticoid hormones such as cortisol diffuse across the plasma membranes of GC-sensitive cells (step 1). Cortisol binds GR in the cytoplasm (step 2). Cytoplasmic GR is bound to two molecules of hsp90 as well as other proteins that are necessary to maintain it as a silent receptor that has high affinity for GC. Cortisol binding initiates a transformation of GR that promotes its dimerization (step 3) and translocation of GC-GR complexes into the nucleus (step 4). In the nucleus, the GC-GR complexes bind with high affinity to DNA sequence motifs (the GREs) in the promoter regions and other regulatory regions of GC-responsive target genes (step 5). Once bound, ligand activated GR transcriptionally regulates these genes (activation of the target gene is shown in steps 6-8 of Figure 1.2). Ultimately, GR activation leads to changes in cellular phenotype and function (step 9) by altering expression of target genes. The type and rate of transcriptional regulation by GR is dependent on protein-ligand interactions and interaction of the GC-GR complex with the basal transcription machinery, co-activators (or suppressors), and other transcription factors (Guido et al., 1996). Changes in cellular phenotype and function by GC-activated GR have been implicated to play important roles in altered leukocyte functions, and especially in innate immunity in cattle and other species (see section IV).

C. Glucocorticoid Agonists and Antagonists

The most important natural agonist for GR is its ligand, cortisol (corticosterone in rodents). At basal blood concentrations (bovine, 4-8 ng/ml; Goff et al., 1989; Smith et al.,1973), cortisol binds with high affinity to the mineralocorticoid receptor and with low affinity to GR. However, when blood cortisol concentrations are increased in response to

stress, the hormone binds with very high affinity to GR (parturition, 15-25 ng/ml; Goff et al., 1989; Smith et al.,1973) (Figure 1.2).

To better understand the roles of GC in cellular responses to stress, specific molecular probes for GR have been developed and used both *in vitro* and *in vivo* (Argawal, 1996; Gagne et al., 1985; Gagne et al., 1986; Rupprecht et al., 1993). Several synthetic GC agonists and antagonists are available commercially. Of particular interest in regard to the current series of studies is the agonist, dexamethasone (DEX), because it binds GR with high specificity and affinity (15-fold higher affinity than cortisol in humans) (Murakami et al., 1979). Dexamethasone has been utilized in studies to visualize GR by conjugating it with identifiable markers, such as radioisotopes or fluorochromes. Fluorescently labeled DEX was utilized for the studies reported in this thesis (Refer to Chapters Two, Three, and Four). The GR antagonist, mifepristone (RU486), is a potent antiglucocorticoid with at least 3-times higher affinity for GR than DEX (Argawal, 1996; Gagne et al., 1985). RU486 successfully competes for GC-binding sites and alters GC-sensitivity of cells by interfering with various stages of GR activation. Progesterone is another antiglucocorticoid that successfully competes with cortisol and dexamethasone for GR (Rupprecht et al., 1993). In the present studies, both RU486 and progesterone were used as antagonists in competition assays with fluorescently-labeled DEX to validate our novel method for visualizing and monitoring bovine leukocyte GR expression (Chapters Two, Three, and Four).

II. GLUCOCORTICOID RECEPTOR EXPRESSION IN LEUKOCYTES

GCs are involved in physiological regulation in almost every tissue of the body. Therefore, human and rodent GR has near ubiquitous tissue distribution, including peripheral blood leukocytes (Ballard et al., 1974). In the past, the most common method to detect and quantitate cytoplasmic GR has been through the use of receptor binding assays, using ^{3}H-DEX and whole cells or cytosolic fractions of cells, followed by Scatchard analysis (Scatchard et al., 1949). Ballard et al. (1974) used this method to determine GR

presence in a variety of mammalian tissues; however, peripheral blood leukocytes were not included in that study. Since then, GR has been detected in circulating human lymphocytes and monocytes by several research groups, but there is some discrepancy in actual numbers of receptors reported for these cells types (See Table 1.1). There is no clear answer to account for the discrepancies in reported numbers of GR in human mononuclear leukocytes except that each group of researchers used slightly different methods to estimate GR (Table 1.1). Nonetheless, one consistency across studies that investigated both lymphocytes and monocytes (reported in Table 1.1) was that lymphocytes had about half the number of GR molecules as monocytes (Distelhorst and Benutto, 1981; Lippman and Barr, 1977). With the exception of an experiment that used cycling female cattle (Martinez et al., 1998), relative expression of GR in bovine leukocytes during physiologically and (or) pharmacologically relevant GC challenges has not been studied. Therefore, the work of the current thesis research seemed logical because glucocorticoids have profound effects on bovine leukocyte phenotypes, functions, and susceptibility to infectious diseases (see below).

Table 1.1 – Summary of glucocorticoid receptor expression in different leukocyte types.

Author(s)	Assay Description	Species	Lymphocyte # GR/Cell	Monocyte # GR/Cell
Lippman and Barr (1977)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	T-Lymphocytes 3130 ± 1316 B-Lymphocytes 2892 ± 1068	7009 ± 1455
Bloom et al. (1980)	Scatchard analysis of [^{3}H] DEX binding in whole cells in bovine leukemia virus-infected cows	Bovine	5000-6000[2]	NA
Distelhorst and Bennutto (1981)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	7200 ± 400	18300±2200
Schlecte et al. (1982)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	6617 ± 979	NA
Junker (1983)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	7400 ± 1560	NA
Kontula et al. (1983)	Scatchard Analysis of [^{3}H] DEX or [^{3}H] medroxyprogesterone binding in whole cells	Human	~4500 for both steroids[2]	NA

Table 1.1 Cont'd

Author(s)	Assay Description	Species	Lymphocyte # GR/Cell [1]	Monocyte # GR/Cell
Shipman et al. (1983)	Scatchard analysis of [^{3}H] DEX binding of cytosolic fractions	Human	4668	NA
Petrichenko et al. (1989)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	4800 ± 460	NA
Sigal et al. (1993)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	6000 ± 393	NA
Molijn et al. (1995)	Scatchard analysis of [^{3}H] DEX binding in whole cells	Human	6352 ± 310	NA
Martinez et al. (1998)[3]	Scatchard analysis of [^{3}H] DEX binding in whole cells	Bovine	Estrogenic stage 5429 ± 1489 Progesteronic stage 7491 ± 515	NA
Weyts et al. (1998)[4]	Scatchard analysis of [^{3}H] DEX binding in whole cells	Carp	492 ± 90	

[1] = Indicates # of receptors in total lymphocytes unless otherwise indicated
[2] = Standard errors not always reported
[3] = Only study to report neutrophil GR per cell; Estrogenic stage = 3924 ± 1221, Progesteronic stage = 2809 ± 459
[4] = Study done on peripheral blood leukocytes

III. THE IMMUNE SYSTEM

As in other mammals, the bovine immune system is composed of two separate but connected defense mechanisms; the innate and adaptive immune systems (See Figure 1.3) (Janeway and Travers, 1996, McCullough and Martinod, 1987). The innate immune system (Figure 1.3, blue text and arrows) is always present and can respond immediately, but with limited repertoire of defense mechanisms, to extracellular micro-organisms. It is activated immediately upon encounter with foreign antigens (such as bacteria), without previous stimulation, and functions using serum protein components in conjunction with phagocytic and killing abilities of neutrophils and monocytes (white blood cells that differentiate into macrophages). Neutrophils are particularly important in defense against numerous infectious diseases of dairy cows such as mastitis and metritis. Indeed, neutrophils are primarily responsible for clearing bacterial infections, especially coliforms, in the mammary gland (Burvenich et al. 1994). However, when neutrophils malfunction and (or) an infection becomes too great a challenge for these phagocytes, the adaptive immune system becomes activated to help target and clear the infecting agent (Figure 1.3, black text and arrows).

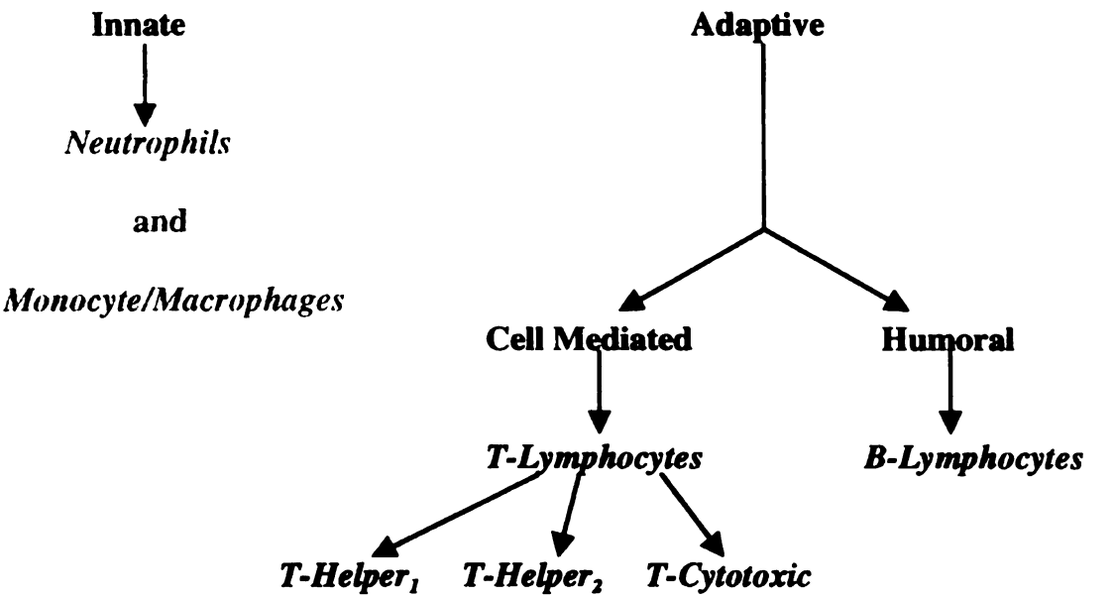


Figure 1.3 – Leukocytes, or white blood cells, are divided into the innate and adaptive immune systems. The innate immune system is indicated by blue text and arrows, and the adaptive immune system by black tesxt and arrows. The lukocutes include (italicized), neutrophils, monocyte/macrophages, and B and T lymphocytes.

Adaptive immunity, while much more specific than innate immunity, requires precise and regulated activation to be effective against infecting organisms. The adaptive immune system is divided into two main categories of immunological defense: humoral immunity and cell-mediated immunity (Figure 1.3, black text and arrows). Humoral immunity is mediated by soluble antibodies that are produced by antigen-activated B-lymphocytes. Antigen-activated T-lymphocytes are responsible for regulating both humoral and cell-mediated immunity. The T-lymphocytes differentiate into subpopulations of $CD4^+$ helper T-lymphocytes (T_H1 and T_H2) and $CD8^+$ cytotoxic T-lymphocytes (T_c) (Figure 1.3).

The specific T-helper lymphocytes connect the innate (Figure 1.4, blue text) and adaptive immune systems (Figure 1.4, black text) allowing the immune system to operate quickly and efficiently to erradicate foreign antigens (Figure 1.4). Helper T-lymphocytes assist other immune cells to function more efficiently by producing a variety of specific cytokines (Figure 1.4, light blue text) at the sites of antigen activation. For example, T_H1 cells (Figure 1.4, green text and arrows) produce IFN-γ which up-regulates major histocompatability complex (MHC) antigen expression by macrophages and increases the killing abilities of phagocytic cells, as well as IL-2, which activates T_C cells. The T_H2 cells (Figure 1.4, red text and arrow) produce IL-4 to activate B-lymphocytes for antibody production, and IL-10 which suppresses T_C cells. Cytotoxic T-cells are extremely effective in eliminating intracellular pathogens, in particular viruses, by causing death of the infected host cell. There is also further interaction between the innate and adaptive immune systems once antibodies are secreted from B-lymphocytes. Opsonization (Figure 1.4, purple text and arrows) occurs when antibodies bind to a specific pathogen, and the F_C region of the antibody is recognized by F_C receptors on the phagocytic cell to facilitate recognition and elimination of the pathogen (Figure 1.4, purple lines). Therefore, the adaptive immune system, and its assistance to the innate immune system, is crucial for the elimination of intracellular, intravesicular, and extracellular pathogens. Related to mastitis-susceptible

dairy cows, a breakdown in the adaptive immune system will directly affect the ability of the innate immune system to eliminate bacterial infections, especially mastitis-causing pathogens, by reducing the effectiveness and efficiency of pathogen recognition and elimination. Interestingly, elevated GC concentrations, whether by natural or artificial causes, alters the mentioned functions and phenotypes of leukocytes leading to generalized immunosuppression and increased disease susceptibilty.

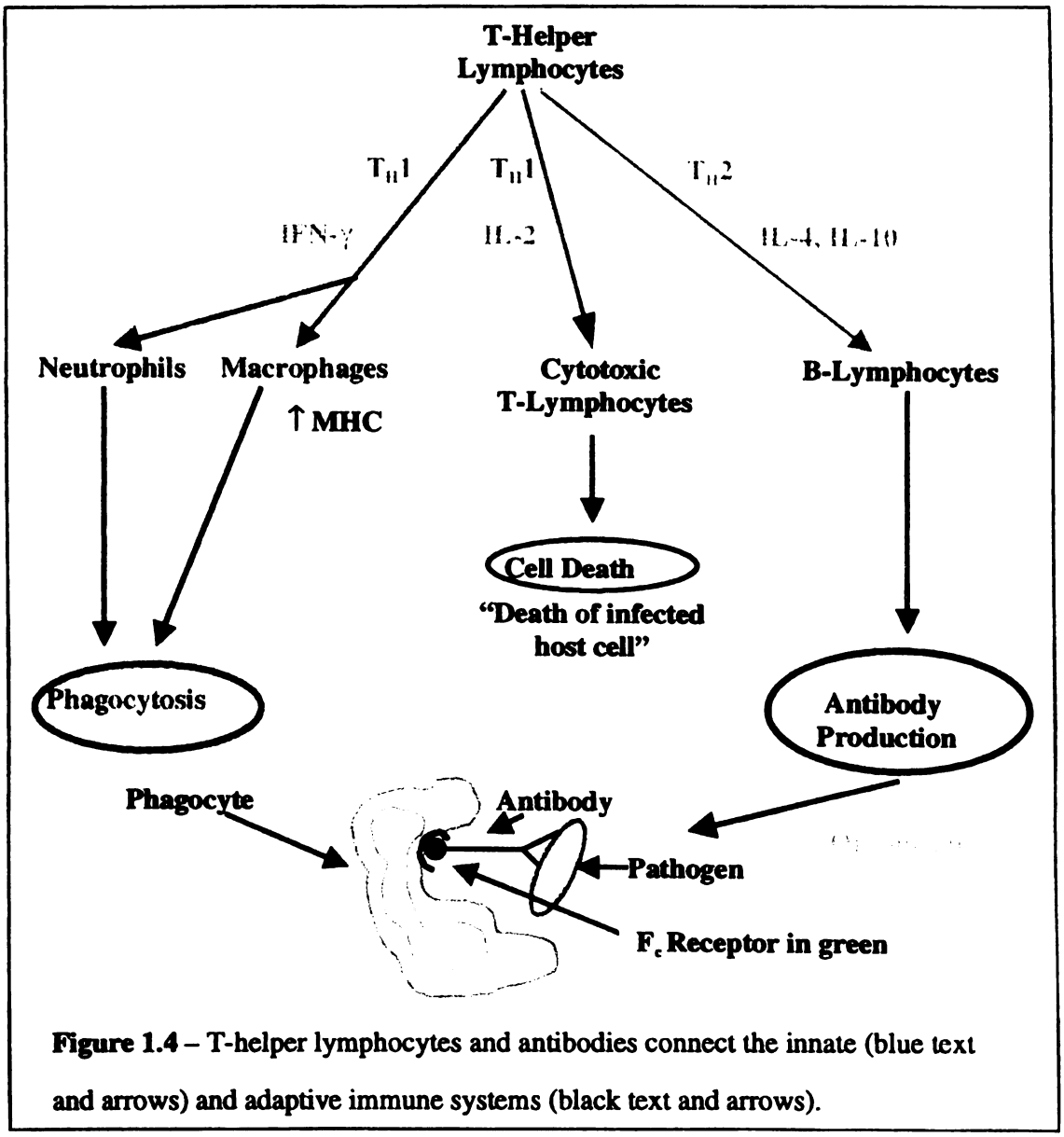


Figure 1.4 – T-helper lymphocytes and antibodies connect the innate (blue text and arrows) and adaptive immune systems (black text and arrows).

IV. IMMUNOSUPPRESSION MODELS

In order to better understand the role that GR may play in immune dysfunctions, effects of GC in both experimental and natural challenge models have been studied. From the following summary of this research, it becomes evident that glucocorticoids, and the glucocorticoid receptor, may be major regulators of immunity and health in cattle.

A. Experimental Glucocorticoid Challenge Model

Evidence has been compiled in studies using DEX-treated mammals that leukocyte functions and phenotypes are dramatically impaired when blood GC concentrations are elevated beyond basal levels. Table 1.2 provides a summary of DEX-induced leukocyte dysfunctions in cattle, sheep, pigs, and humans. Dexamethasone consistently causes leukocytosis (Paape et al., 1981; Gwazdauskas et al., 1980) along with impaired leukocyte functions. Neutrophils of the innate immune system are most profoundly affected by DEX. For example, they lose the ability to marginate along the endothelium and to migrate from blood into infected tissue due to down-regulation of the adhesion molecules, CD62L and CD18 *in vivo* (Burton et al., 1995). This is contradictory to the increase in random migration seen *in vitro*, however this is probably due to the type of assay used, where neutrophils are under agarose and able to move in a non-directed motion whether they have CD62L and CD18 expression or not (Roth and Kaeberle., 1981; Roth et al., 1982). In turn, the down-regulation of CD62L and CD18 leads to neutrophilia and increased susceptibility to diseases caused by bacterial infections. This is important in dairy cows because neutrophils are the first line of defense against mastitis-causing bacteria in the mammary gland. Burton and Kehrli (1995), and Wesley et al. (1989), highlighted the detrimental effects of DEX administration on health of cows, which experienced dramatic increases in bacterial shedding in milk of experimentally-infected mammary glands and a shift from subclinical to clinical mastitis. Table 1.2 also implicates a role for GC and GR in adaptive immunity. These findings, along with others listed in Table 1.2 strongly

implicated a role for GCs in disease susceptibility of stressed cows and lead to our hypothesis that GC-activated receptors (GR) regulate the function and phenotype of bovine leukocytes. If true, natural situations of stress, such as parturition, should also respond by causing GC-activation of GR which in turn may alter leukocyte functions and phenotypes related to immunosuppression.

Table 1.2 – Summary of leukocyte dysfunctions resulting from administration of glucocorticoids into cattle, sheep, pigs, and humans.

Leukocyte Type		Dysfunction	Species	Citation
Neutrophil	↑	Blood neutrophil counts	Cattle-Calf Cattle Cattle Humans	Carlson and Kanenko, 1976 Roth et al., 1982 Gwazdauskas et al., 1980 Stossel, 1974
	↓	Margination	Cattle	Carlson and Kanenko, 1976
	↑	Random migration *in vitro*	Cattle Cattle	Roth and Kaeberle, 1981 Roth et al., 1982
	↓	Bacterial ingestion by phagocytosis	Cattle Cattle	Paape et al., 1981 Roth et al., 1981
	↓	Oxidative metabolism	Cattle	Roth and Kaeberle, 1981
	↓	Antibody dependent cell-mediated cytotoxicity	Cattle	Roth and Kaeberle, 1981
	↓	CD62L and CD18 expression *in vivo*	Cattle Human	Burton and Kehrli, 1995 Filep et al., 1997
	↑	Bacterial shedding in milk – *S. aureus* *-L. monocytogenes*	Cattle Cattle	Burton and Kehrli, 1995 Wesley et al., 1989
Monocyte/ Macrophage	↓	Phagocytic capabilities	Cattle	Roth and Kaeberle, 1982

Table 1.2 continued

	↓	MHC II expression	Cattle Humans	Burton and Kehrli, 1996 Celada et al., 1993
	↓	Cytokine expression –TNF-α, IL-1, IL-6, IL-12	Human	Almawi et al., 1996 – review
Lymphocyte	↓	Lymphocyte proliferation	Sheep Humans	Khosraviani and Davis, 1996 Kontula et al., 1983
	↓	Lymphocyte blastogenesis in response to PHA and ConA	Cattle Sheep Pigs	Roth et al., 1982 Staples et al., 1983 Flaming et al., 1994
	↓	T-Lymphocyte cytokine expression - IFN-γ - IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IFN-γ - IFN-γ	 Cattle Human Human	 Nonnecke et al., 1997 Almawi et al., 1996 – review Arya et al., 1984
	↓	MHC I expression	Cattle Humans	Burton and Kehrli, 1996 Celada et al., 1993
	↓	IgM Secretion	Cattle	Nonnecke et al., 1997
	↑	γδ T-lymphocyte migration	Cattle	Burton and Kehrli, 1996

B. Natural Glucocorticoid Challenge Model

Periparturition in dairy cows presents numerous stressful challenges including metabolic, nutritional, physical, psychological, and disease. Indeed, high disease susceptibility of periparturient dairy cows led bovine immunologists to study immune functions and the role of stress in their dysfunctions. Periparturition is defined as the period of 3 weeks before to 3 weeks after calving. During this time, daily feed intake is

reduced, tissue sensitivity to insulin decreased, there are wide fluctuations in blood hormone concentrations, milk production and secretion is initiated, negative energy balance ensues, and the animal gives birth to a calf. Approximately 3 days before calving, concentrations of cortisol in maternal blood begin to increase from basal levels of 4 to 8 ng/ml and peak at 15-25 ng/ml at calving (Goff, et al., 1989; Guidry et al., 1976). Therefore, it is not surprising that periparturition is associated with the same debilitating effects on leukocyte functions and phenotypes as occurred when GC was administered directly. Table 1.3 outlines the studies in periparturient dairy cows that have linked parturition and the natural, and dramatic increase of GCs with leukcocyte dysfunctions. Most of these studies used neutrophils, most likely due to their importance in combating bacterial infections in the mammary gland that lead to mastitis. Considering that mastitis costs the US dairy industry upwards of 2 billion dollars annually in discarded milk (National Mastitis Council, 1996), a priority among mastitis researchers is to elucidate the mechanisms that lead to parturition-induced leukocyte immune dysfunctions. Our own priority was to begin to study bovine GR as a possible mediator of cortisol induced immune dysfunctions in periparturient dairy cows.

Table 1.3 – Summary of Leukocyte Dysfunctions in Periparturient Dairy Cows

Leukocyte Type		Dysfunction	Citation
Neutrophil	↑	Blood neutrophil counts	Lee and Kehrli, 1998 **Preisler et al, 1999**
	↑	Random migration *in vitro*	Kehrli et al., 1989a Nagahata et al., 1988
	↓	Bacterial ingestion by phagocytosis *in vitro*	Kehrli et al., 1989a
	↓	Oxidative metabolism *in vitro*	Kehrli et al., 1989a Moreira da Silva et al., 1998

Table 1.3 continued

	↓	Antibody dependent cell-mediated cytotoxicity *in vitro*	Cai et al., 1994 Kehrli et al., 1989a
	↓	CD62L and CD18 expression *in vivo*	Lee and Kehrli, 1998
Monocyte/ Macrophage		NA	NA
Lymphocyte	↓	Lymphocyte blastogenesis in response to PHA and ConA *in vitro*	Kehrli et al., 1989b Saad et al., 1989
		Changes in T-lymphocyte subsets *in vivo*	Van Kampen and Mallard, 1997
	↓	Antibody producing activity *in vitro*	Nagahata et al., 1992

Given the information presented in Tables 1.2 and 1.3, the overall hypothesis of this thesis is that bovine leukocytes contain cytoplasmic GR and have altered expression of GR during periparturition in relation to the natural increase in cortisol. To test this hypothesis specific, objectives were designed:

Objective 1: Determine whether bovine leukocytes express GR (Chapter two).

Objective 2: Determine if there is differential expression of GR in different bovine leukocyte types (Chapter two).

Objective 3: Test the effects of parturition on leukocyte expression of GR in dairy cows (Chapters three and four).

Objective 4: Correlate leukocyte GR expression with blood cortisol concentrations in periparturient dairy cows (Chapters three and four).

Objective 5: Correlate blood cortisol and leukocyte GR expression with circulating numbers of each leukocyte type (Chapters three and four).

Results of these studies could implicate GC-activated GR as a regulator of altered leukocyte phenotypes and functions, which may lead to immunosuppression and increased disease susceptibility in periparturient dairy cows. The importance of these findings would indicate that GR is a key protein in immunoregulation that could become a target for the design and development of novel pharmaceuticals intended to prevent or treat parturition-induced diseases. This would also have implications for treatments in other natural stress situations, not only in dairy cattle, but in other production livestock species as well.

CHAPTER TWO

Development and Validation of an Assay to Monitor Glucocorticoid Receptor Expression in Bovine Blood Leukocytes

I. ABSTRACT

One objective of this study was to develop a fluorescent staining assay to detect cytoplasmic leukocyte GR expression by using fluorescence-activated flow cytometry. Whole blood was routinely collected from three different randomly sampled Holstein cows to allow test samples to be run in triplicate during assay development and validation. Glucocorticoid receptor expression was detected using fluorescein isothiocyanate- (FITC) labeled dexamethasone (DEX). Briefly, 5×10^5 leukocytes were fixed and permeablized and then incubated with either 1×10^{-5} and 5×10^{-5} M of FITC-DEX. These concentrations of the GR probe were determined to saturate leukocyte GR in validation studies. Following 3 washes in PBS that contained 5% ethanol, FITC fluorescence intensity in leukocytes was determined flow cytometrically. Fluorescence microscopy demonstrated that the FITC label was located in the cytoplasm of leukocytes. Confidence in this assay to detect and monitor GR expression was achieved by high repeatability of results over 3 consecutive days for leukocytes of the 3 sampled cows (variation due to sampling error < 10%). Furthermore, our demonstration that FITC fluorescence intensity was significantly reduced ($P < 0.05$) when progesterone and mifepristone (RU486) were used at 1000 fold excess to compete in the assay with FITC-DEX validated that DEX was binding to GR.

A second objective of this study was to determine GR expression in blood leukocytes differentiated into neutrophils, monocytes, and lymphocytes. Cell surface expression of CD45 was used as the leukocyte differential. Anti-bovine CD45 monoclonal antibody and a secondary antibody conjugated with phycoerythrin (PE) were used to stain the plasma membranes of $5x10^5$ leukocytes prior to their fixing, permeabilization, and GR staining using FITC-DEX. Dual-labeled (CD45 and GR) cells were again analyzed by fluorescence-activated flow cytometry, and validated by fluorescence microscopy. Based on flow cytometric analysis of these dual-labeled cells, CD45 staining in combination with side-scatter properties clearly differentiated leukocytes into lymphocytes, monocytes, and neutrophils. Microscopy demonstrated that GR staining was predominantly cytoplasmic and that monocytes and neutrophils stained with greater FITC intensity than lymphocytes. In fact, analysis of the mean FITC fluorescence intensity of these 3 leukocyte populations showed that monocytes had twice as much GR expression as lymphocytes, with neutrophils falling in between. Therefore, based on the assay developed and validated in this study, it was concluded that CD45 is an effective leukocyte differential in cattle and that bovine monocytes and neutrophils contain higher levels of cytoplasmic GR than bovine lymphocytes. An important implication of this observation is that bovine monocytes and neutrophils may be more sensitive to increases in blood glucocorticoid concentrations than lymphocytes. If true, this would suggest that innate immunity is more susceptible to stress than acquired immunity in cattle.

II. INTRODUCTION

Glucocorticoids are adrenal cortex steroid hormones that have crucial involvement in stress responses that can directly affect a cell's phenotype and function (Norman and Litwack, 1997). This is striking for leukocytes, the vital cells of the immune system (DeRijk and Berkenbosch, 1994.). Actions of GC are mediated by their binding to intracellular GR that are located in the cytoplasm of target cells, including blood leukocytes. Upon activation by GC, GRs dimerize, translocate into the nucleus, bind to DNA in regulatory regions of target genes, and regulate transcription of those genes (Bamberger et al., 1996; Oakley and Cidlowski, 1993). Therefore, GR is called a ligand-activated transcription regulator. Transcriptional regulation by GC-activated GR can be in one of two directions, activation (up-regulated gene expression) or repression (down-regulated gene expression). In the immune system of most species, including cows, GCs tend to mediate repression rather than activation of most immune response genes (Refer to Tables 1.2 and 1.3 in Chapter One).

Cortisol (corticosterone in rodents) is the major natural GC in mammals. In situations of perceived stress, blood cortisol concentrations elevate beyond basal levels resulting in high affinity binding of cortisol to GR. This initiates predominantly repression of transcription of genes responsible for leukocyte trafficking, migration, antigen presentation, and innate and acquired immune responses (Burton and Kehrli, 1995; Burton et al., 1995; DeRijk and Berkenbosch, 1994; Lee and Kehrli, 1998; Wilckens and De Rijk, 1997). The number of GR molecules expressed in the cytoplasm of cells directly affects the degree of responsiveness (gene transcription) of cells to GC. This implies that differential GR expression among different cell types will determine

susceptibility of those cells to stress. It also implies that changes in GR expression in any one cell type will alter the cell's responsiveness to stress. Therefore, it seemed relevant to explore differential expression of GR in bovine blood neutrophils, monocytes, and lymphocytes (this Chapter) and to monitor potential changes in GR expression in these leukocyte types during periods of natural increases in blood glucocorticoid concentrations, such as occurs during parturition (Chapters Three and Four).

Current methods to detect GR protein expression include the use of species specific polyclonal and monoclonal antibodies as well as binding of radiolabeled dexamethasone (DEX) to cytosolic fractions or whole cells (Refer to Table 1.1 in Chapter One). Dexamethasone is a synthetic GC that binds with high affinity and specificity to the steroid-binding domain of GR. Dexamethasone is a GC agonist because it binds GR and carries out the functions of GC as if it were the natural GR ligand. Study of the bovine GR has been relatively slow compared with human GR because the DNA sequence of bovine GR cDNA and gene are not known. Furthermore, bovine GR-specific antibodies and molecular probes needed to study the physiology of GR are not available. However, DEX is available in both radiolabeled and fluorochrome-conjugated forms and can be used for preliminary assessment of expression and regulation of bovine GR. The objective of this study was to use fluorescently-labeled DEX in conjunction with appropriate GC antagonists to develop and validate an assay to enable us to study bovine GR in intact bovine blood leukocytes. Fluorescein isothiocyanate (FITC)-conjugated DEX, progesterone, RU486, fluorescence-activated flow cytometry, and fluorescence microscopy were used to accomplish this objective.

III. MATERIALS, METHODS, and RESULTS

A. Development of an Assay to Monitor Bovine Leukocyte GR

1. *Blood Collection, Leukocyte Counting, and Standardization of Cell Numbers for GR Staining*

Reagents, supplies, equipment and solutions used in the development and validation of the assay to detect cytoplasmic leukocyte GR are listed in Appendix Table 1 and are indicated in this Chapter and in the remainder of this thesis by bracketed ([ ]) letters. In order to study leukocytes, whole blood samples were aquired from a random sample of Holstein cows housed at the Michigan State University Dairy Teaching and Research Facility (chosen based on their availability). During each sampling period, 3 animals were selected to allow the assay to be run in triplicate. Blood samples for leukocyte counting, flow cytometric analysis, and fluorescence microscopy of neutrophil, lymphocyte, and monocyte GR expression were collected by tail veinupuncture into Vacutainer brand collection tubes containing 1.0 ml acid citrate dextrose anticoagulant [p, q] using 20 *g* 2.5 cm multi-sample needles [o].

Total leukocytes were counted to standardize the number of cells added to assays for monitoring GR expression. First, 5 ml of whole blood from Vacutainer tubes containing acid citrate dextrose [q] was added to 50 ml polystyrene conical tubes [s]. Red blood cells were lysed for 1.5 min using 10 ml cold hypotonic lysing solution [cc] (10.56 m*M* Na_2HP0_4, 2.67 m*M* NaH_2PO_4, pH 7.3). This was followed immediately by the addition of 20 ml cold hypertonic restoring solution [dd] (10.56 m*M* Na_2HPO_4, 2.67 m*M* NaH_2PO_4, 0.43 *M* NaCl, pH 7.3) to restore the isotonicity to the remaining cells. Remaining leukocytes were then pelleted by centrifugation (1000 x *g* [y]) for 5 min at 22^0

Figure 2.1 – Ninety-six well plate map design, developed for the assay to detect leukocyte glucocorticoid receptors.

	1 Auto- fluorescence	2 FITC Negative Control	3 PE Negative Control	4 Single Color (PE)	5 Single Color (FITC)	6 PE + FITC-DEX 1 x 10-5 M	7 PE + FITC-DEX 5 x 10-5 M	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

C) and the supernatants were aspirated. The cell pellet was washed twice in 2 ml of cold PBS [ee] and centrifuged (800 x *g* for 5 min at 22^0 C). Leukocytes were suspended in 5 ml of PBS, counted on a hemacytometer [z], and adjusted to 5 x 10^6 cells/ml in PBS. Then, 100 ul of cells (5 x 10^5 cells) were added to appropriate wells of a 96-welled, U-bottom microtiter plate [t,u] and subjected to fluorescence staining (below).

2. *Dual-Color Fluorescent Labeling of Leukocytes*

For each animal sampled, 7 wells of prepared leukocytes (above) were used for fluorescent staining of blood leukocytes (See Plate Map in Figure 2.1). The first well was a negative control (autofluorescence; 100 ul of PBS added). The second well was for the FITC fluorochrome negative control [d] (1:100 dilution in PBS of irrelevant anti-mouse IgG_1 antibody conjugated with FITC). The third well was for the PE fluorochrome negative control [g] (1:100 dilution of irrelevant PE-conjugated IgG_{2a} antibody added). The fourth well was for the single-color, PE-stained cells [i, e] (100 ul of 5 ul/ml dilution of primary anti-CD45 antibody added, followed by 100 ul of a 1:400 dilution of PE-conjugated anti-IgG_{2a} secondary antibody). The fifth well was for the single-color, FITC-DEX stained cells [c] (100 ul of 1 x 10^{-5} *M* of FITC-DEX added). The sixth and seventh wells were designated for dual-color staining that included both PE (CD45; [i, e]) and FITC-DEX (GR [c]).

For the dual-staining protocol, CD45 labeling with PE was completed first, followed by GR labeling with the FITC-DEX. The CD45 staining protocol started with a 30 min incubation (4^0 C in the dark) of leukocytes in anti-CD45 antibody [i] (wells 4, 6,

7; remaining wells received 100 ul of PBS). The reaction was slowed by the addition of 100 ul of cold PBS. Plates were then centrifuged (500 x *g* for 5 min at 22^0 C) and supernatants were gently aspirated from each well. Cells were resuspended in secondary antibody [e] (wells 4, 6, and 7), negative isotype control antibody [g] (well 3), or PBS (wells 1 and 2) using a multichannel pipettor [bb, n], and incubated for 10 min at 4^0 C in the dark. Then, 100 ul of cold PBS was added, plates were centrifuged (500 x *g* for 5 min at 22^0 C), and supernatants were aspirated. Leukocytes were washed one more time in 200 ul of PBS to remove unbound secondary antibody.

Once CD45 staining was finished, GR staining was accomplished. To do this, leukocytes were fixed with 150 ul of a 1:10 dilution (in ddH_20) of FACS lysis solution that contained less than 15% formaldehyde [a] for 10 min, and the solution removed after centrifugation at 500 x *g* for 5 min. Next, the leukocytes were permeablized using 50 ul of Solution B [j] for 15 min. This reaction was immediately slowed by adding 150 ul of wash solution [gg] (10 m*M* Tris, pH 7.6, containing 0.1 % DMSO and 5 % ethanol), followed by centrifugation (500 x g for 5 min at 22^0 C) and aspiration of supernatants. Leukocytes were then incubated with 100 ul of either 1 $x10^{-5}$ *M* (wells 5 and 6) or 5 $x10^{-5}$ *M* (well 7) of FITC-DEX to label GR [c], or with the FITC negative control [d] (well 2). Leukocytes in remaining control wells received only 100 ul of incubation solution [ff] (10 m*M* Tris, pH 7.6, containing and .1 % BSA). Plates were left to incubate for 10 min at 22^0 C in the dark. Following incubation, leukocytes were washed 3 times in washing solution [gg], pelleted by centrifugation (500 x *g* for 5 min at 22^0 C), and finally resuspended in 200 ul sheath fluid [f] for immediate flow cytometric acquisition.

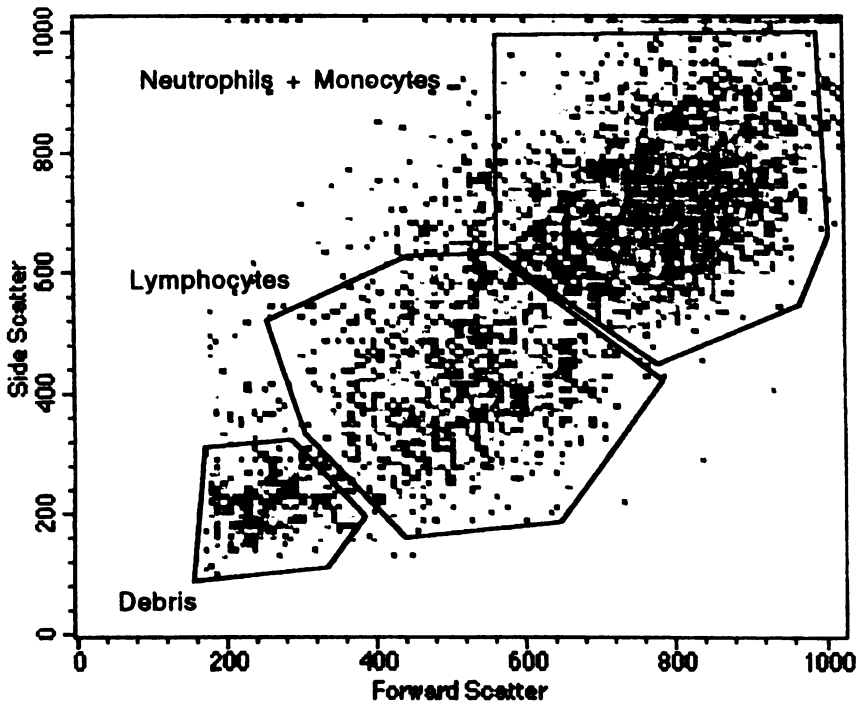


Figure 2.2 – Flow cytometric identification for a representative animal of bovine leukocyte populations based on forward-scatter (x-axis) and side-scatter (y-axis) characteristics. Permeablized cells gated into two regions. The larger more granular cells in the upper right region were designated neutrophils and monocytes. The lower region consisted of the lymphocyte population and was separated from events that were considered debris.

For actual data acquisitions, stained leukocytes were transferred to 12 x 75 mm Falcon brand polystyrene tubes [m], which fit on the sample injection port of the FACSCalibur flow cytometer [w] (see below).

3. *Flow Cytometric Acquisition of Dual-Color Labeled Leukocytes*

A FACSCalibur fluorescence activated flow cytometer [w] and the acquisition package of CellQuest software [v] were utilized to acquire the dual-color labeled leukocyte data. Unstained cells in well 1 (Figure 2.1) were used to check the forward scatter (FSC = size characteristics) and side scatter (SSC = granularity characteristics) parameters of the leukocytes (Figure 2.2). This allowed individual animal variation in leukocyte physical properties to be adjusted in FSC-SSC density plots for each assay run. Based on the single-color leukocytes in wells 5 and 4 (Figure 2.1), the FL-1 (FITC) and FL-2 (PE) detectors were adjusted for optimal demonstration of green and red fluorescence intensities on histograms (respectively), and used to optimize compensation settings for the two-color analyses of leukocytes in wells 6 and 7 (Figure 2.1). FL-1, FL-2, and compensation settings were not altered once they were set. Cells from wells 2 (FITC negative control) and 3 (PE negative control) (Figure 2.1) were used to set background fluorescence intensities. Finally, leukocytes in wells 6 and 7 were collected as the dual-colored CD45/GR data sets used for subsequent hypothesis testing. Five thousand events were acquired from each of these wells for further flow cytometric analyses (see below).

4. *Flow Cytometric Analysis of Dual-Color Labeled Leukocyte Data*

The analysis package of CellQuest software [v] was used to determine GR expression in CD45-differentiated leukocytes. Glucocorticoid receptor expression was recorded as the geometric mean FITC fluorescence intensity (MFI) of the differentiated

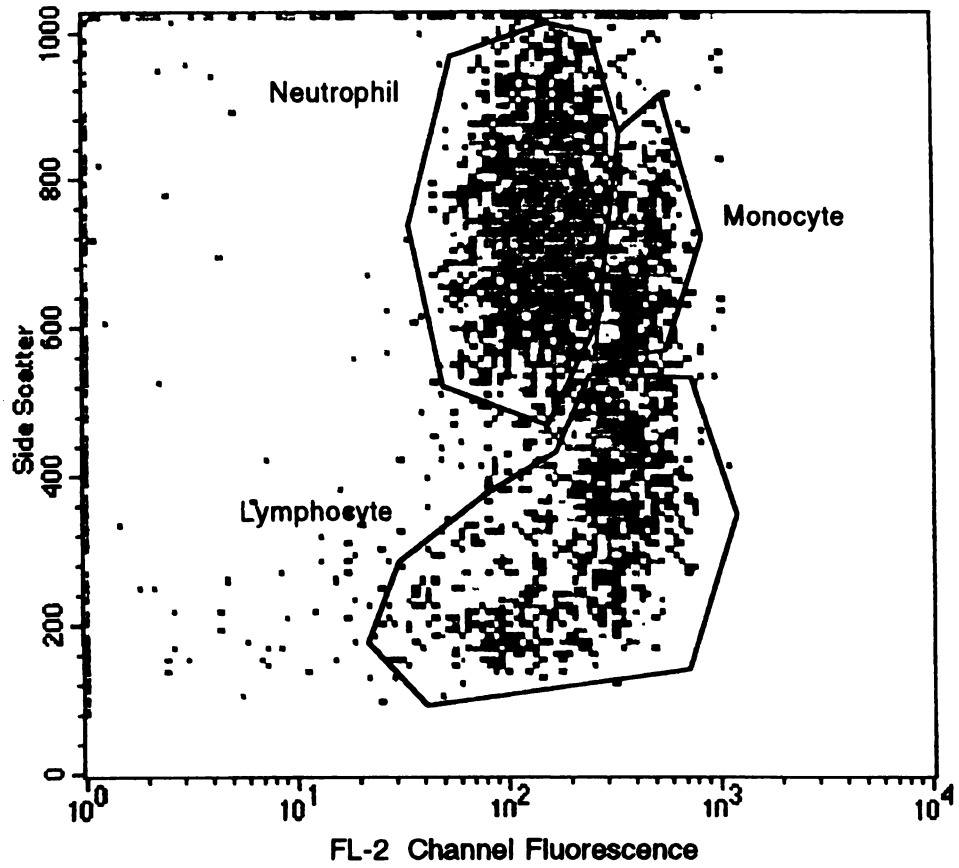
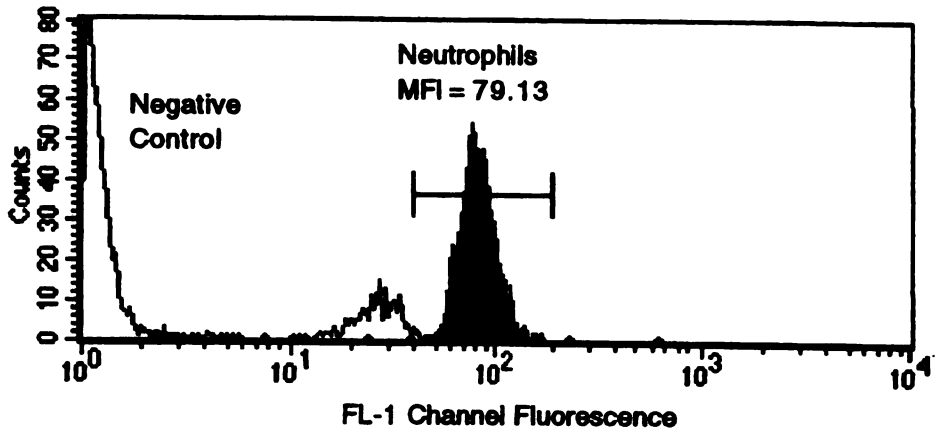


Figure 2.3 – Flow cytometric identification for a representative animal of CD45 differentiated bovine leukocyte populations based on FL-2 Channel Fluorescence (PE intensity; x-axis) and side-scatter characteristics. Using CD45 as a leukocyte differential, neutrophil, monocyte, and lymphocyte populations were distinguishable for future individual flow cytometric analyses of GR expression.

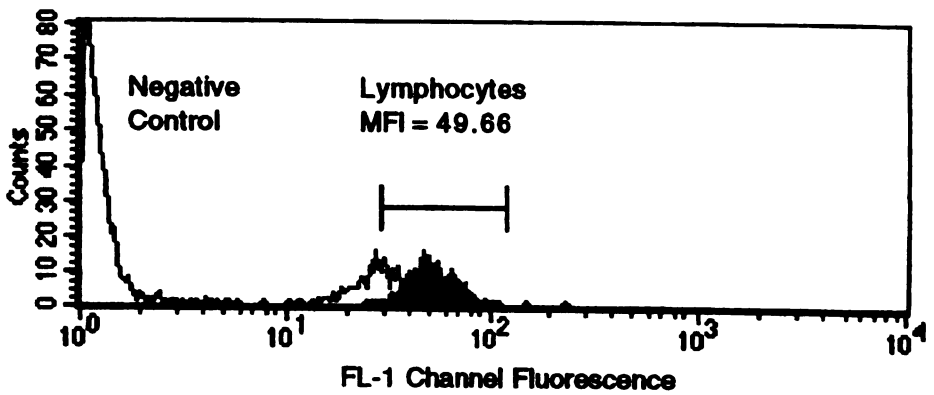
leukocytes. Leukocyte types were differentiated using both side scatter (granularity) and CD45 expression characteristics (Figure 2.3). FL-1 fluorescence histograms were then plotted for each leukocyte type identified as neutrophils (Figure 2.4a), lymphocytes (Figure 2.4b), or monocytes (Figure 2.4c). The MFI values of the fluorescence histograms were used to indicate GR expression by each leukocyte type (Figure 2.4). As summarized in the overlay histograms of Figure 2.5, monocytes from 3 cows had approximately twice the GR expression (mean MFI $\pm$ SEM = 115.72 $\pm$ 11.38) as lymphocytes (mean MFI $\pm$ SEM = 49.25 $\pm$ 2.64), with neutrophil GR expression lying in between (mean MFI $\pm$ SEM = 73.64 $\pm$ 2.8).

Figure 2.4 – GR expression in FITC-DEX stained bovine leukocytes from a representative cow. GR expression was determined by FITC MFI in FL-1 channel fluorescence (x-axis). Neutrophils from this cow stained with a MFI = 79.13 (Figure 4 A), lymphocytes with a MFI = 49.66 (Figure 4 B), and monocytes with a MFI = 117.06 (Figure 4 C).

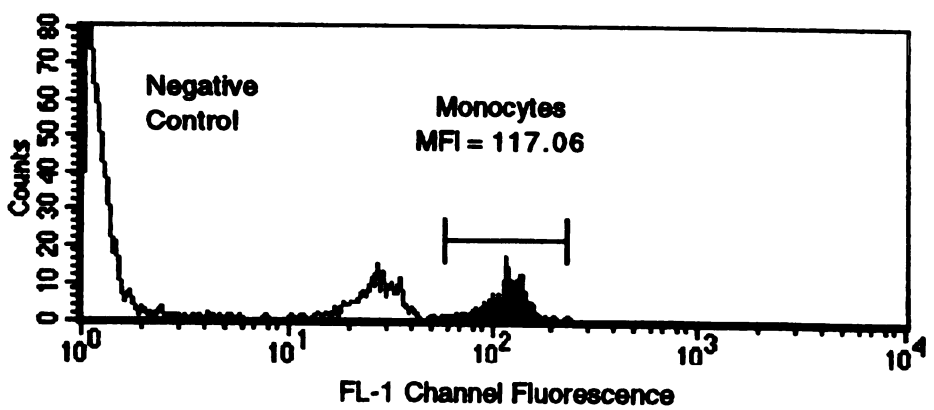
A.


Neutrophils
MFI = 79.13
Negative
Control
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL-1 Channel Fluorescence

B.


Negative
Control
Lymphocytes
MFI = 49.66
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL-1 Channel Fluorescence

C.


Negative
Control
Monocytes
MFI = 117.06
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL-1 Channel Fluorescence

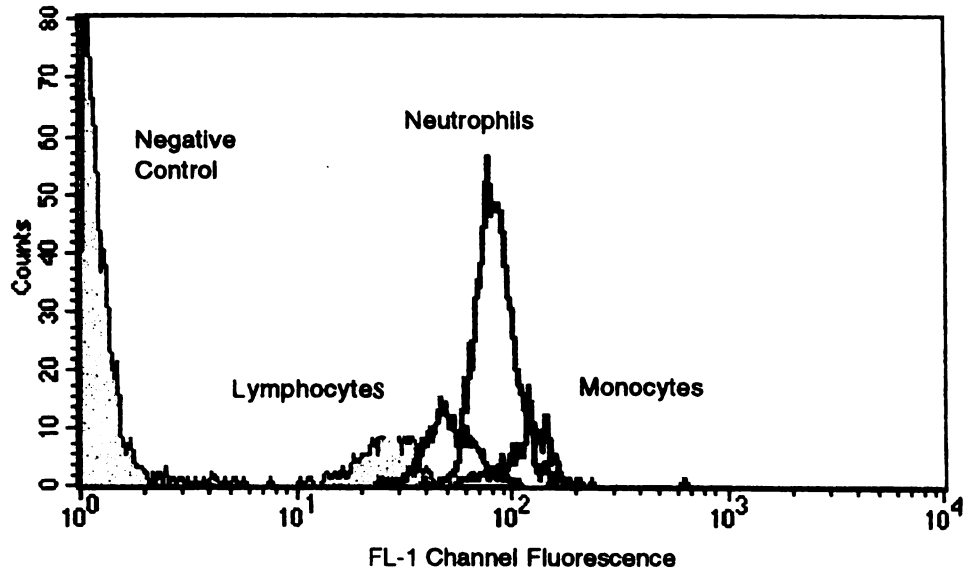


Figure 2.5 – Differential GR expression in FITC-DEX stained bovine leukocytes was determined by FITC-MFI in FL-1 channel fluorescence (x-axis). Shown in this histogram overlay are representative peaks for each leukocyte type from a representative animal. Monocytes express approximately twice as much GR as lymphocytes, and neutrophil GR expression lies in between.

B. Validation of the Assay Developed to Monitor Bovine Leukocyte GR

To arrive at the final dual-color labeling protocol described above, several important issues of assay protocol and validation were addressed during development. The GR staining protocol using FITC-DEX was developed by testing FITC-DEX concentration (GR saturation), permeablization and washing procedures, repeatability of MFI results among 3 cows over 3 consecutive days, and specificity of FITC-DEX for GR labeling. Part way through the GR staining assay protocol, it was observed that permeablized leukocytes had dramatically altered physical characteristics compared with non-permeablized cells, such that monocytes could not be distinguished from lymphocytes and neutrophils based simply on FSC-SSC characteristics (Figure 2.2). Therefore, the GR staining protocol had to be adapted to accommodate the CD45 leukocyte differential staining (Figure 2.3). Finally, it was pertinent that we show which cellular component was staining with FITC-DEX, so stained leukocytes were additionally visualized using fluorescence microscopy. The following sections describe details of the assay development and validation processes that resulted in the final dual-color labeling protocol (described above) that was utilized for all subsequent studies reported in this thesis.

1. *Testing Permeablization Solution and Two Washing Procedures to Eliminate Unbound FITC-Dexamethasone*

As described in the literature review, GR are cytoplasmic so it was important that we got FITC-DEX into leukocytes for GR staining and then removed excess unbound

hormone from the cells. Fortunately, DEX is a steroid that can easily enter the cytoplasm of cells. We determined that this process was facilitated by permeablizing the leukocyte plasma membranes with a commercial reagent [j]. Furthermore, the FITC-DEX was kept in solution during incubation and washing procedures by including 5% ethanol in all solutions added to the leukocytes. To eliminate unbound FITC-DEX after GR staining, two washing procedures were tested. Due to the amount of Solution B needed and the large scale of this testing procedure, only 1 animal was used for this large data acquisition. In the final washes of the above GR staining protocol, a 3 times (3x) washing procedure in wash solution [gg] was compared to a 1 hour incubation in the wash solution followed by the 3x washing procedure on both permeablized and non-permeablized cells. This permeablization and washing test was completed using a series of FITC-DEX molar concentrations for GR staining.

As demonstrated in Figure 2.6 for "neutrophils + monocytes" (determined only by FSC-SSC characteristics at this point), flow cytometric analysis following each washing procedure revealed no differences in FITC MFI between 1h + 3x washing and 3x washing, irregardless of concentration of FITC-DEX used to stain GR. However, more unbound FITC-DEX was washed out of the permeablized cells than non-permeablized cells. The same result was obtained for lymphocytes (Table 2.1). Results in Figure 2.6 and Table 2.1 also demonstrate that lower concentrations of FITC-DEX (in the 1:40 to 1:160 range which is between 7.5×10^{-5} M and 1.5×10^{-5} M) would probably be the most appropriate for this assay to stain GR, since MFI values tended to plateau in these FITC-DEX ranges. Because of these results, the 3x washing procedure for permeablized cells was adopted (described earlier) due to its ease and efficiency.

Table 2.1 – Comparison of 3x washing and 1 h plus 3x washing procedures on permeablized and non-permeablized neutrophils (A) and lymphocytes (B) from a representative animal.

(A)

	Non-Permeablized "Neutrophils"		Permeablized "Neutrophils"	
Dilution of FITC-DEX	MFI 3 x Wash	MFI 1 h + 3 x Wash	MFI 3 x Wash	MFI 1 h + 3 x Wash
1:10	161.38	105.63	61.33	64.57
1:20	61.25	68.78	41.49	35.22
1:40	37.03	33.26	18.88	21.14
1:80	22.05	18.89	10.85	13.92
1:160	10.51	11.74	7.02	9.18
1:320	7.59	9.37	5.75	6.66
1:640	4.09	5.41	4.80	4.97

(B)

	Non-Permeablized "Lymphocytes"		Permeablized "Lymphocytes"	
Dilution of FITC-DEX	MFI 3 x Wash	MFI 1 h + 3 x Wash	MFI 3 x Wash	MFI 1 h + 3 x Wash
1:10	60.11	37.43	34.60	32.52
1:20	27.38	23.61	21.80	19.58
1:40	14.76	13.58	9.61	11.17
1:80	7.10	7.43	5.92	7.09
1:160	3.39	4.03	3.28	4.48
1:320	2.40	3.15	2.69	3.19
1:640	1.45	1.88	2.48	2.73

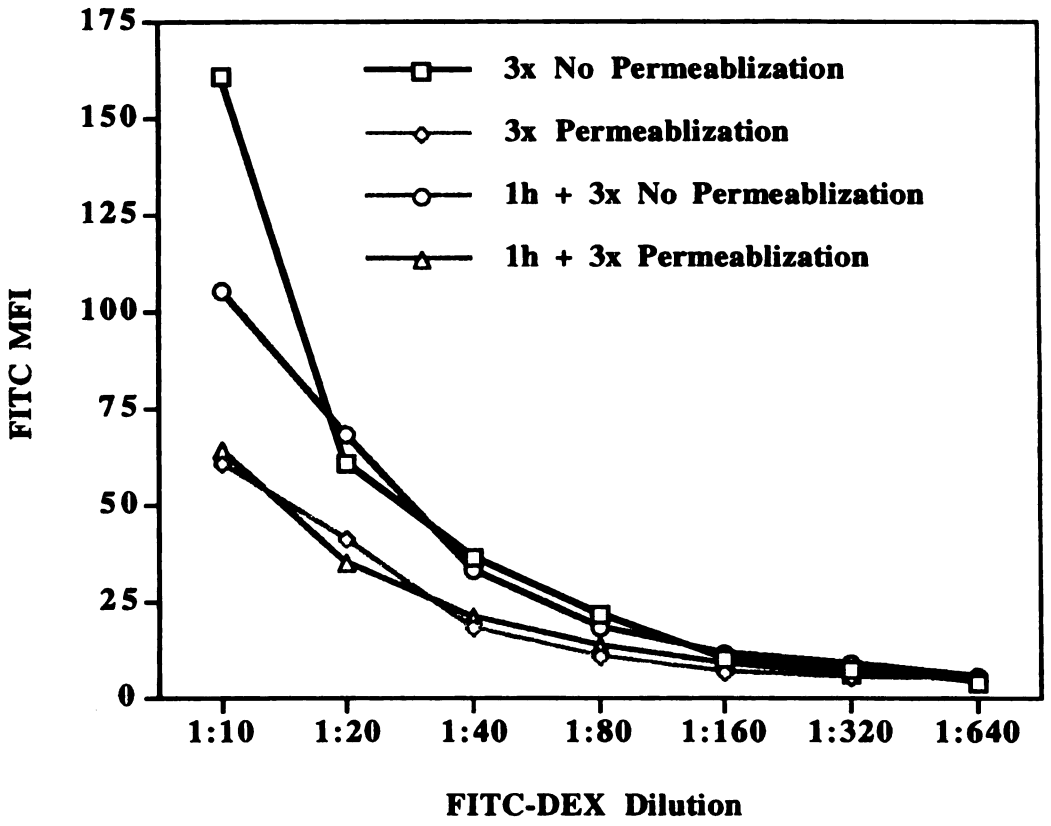


Figure 2.6 – Removal of excess, unbound FITC-DEX requires the use of permeablization solution and a 3x washing procedure. A 3x washing procedure (black and red lines) was tested against a 1 hr + 3x washing procedure (green and blue lines) (data on the "neutrophil + monocyte" population). It was determined that, FITC-DEX is better washed out of permeablized leukocytes (red and blue lines) than of non-permeablized leukocytes (black and green lines), and that a 3x wash was sufficient.

2. *GR Saturation with FITC-Dexamethasone*

To enable us to monitor changes in leukocyte GR expression in subsequent studies (reported in Chapters Three and Four), it was important that leukocytes were stained to the point of receptor saturation. In the tests of washing procedures described previously, doubling dilutions of FITC-DEX were employed and this resulted in a constant change in MFI (Figure 2.6). The objective of this validation experiment was to determine the point of saturation of GR by FITC-DEX when increasing molar concentrations of this probe were used. Accordingly, the following molar concentrations were tested (using permeablized cells and the 3x washing procedure): 1×10^{-6}, 1×10^{-5}, 3×10^{-5}, 4×10^{-5}, 5×10^{-5}, 6×10^{-5}, 7.5×10^{-5}, 1×10^{-4}, and 5×10^{-4}. At the 5×10^{-4} M FITC-DEX concentration, analysis was almost impossible because cells did not remain viable. Nonetheless, saturation curves could be plotted for the FITC-DEX concentrations below 5×10^{-4} M and representative curves for "neutrophils + monocytes" of three cows appear in Figure 2.7. From Figure 2.7, clear plateaus were reached at FITC-DEX concentrations between 4×10^{-5} M and 7.5×10^{-5} M. Based on these results, one concentration within the plateu (5×10^{-5} M) and one just below saturation (1×10^{-5} M) were selected as the concentrations for use in the final dual-labeling protocol (described previously).

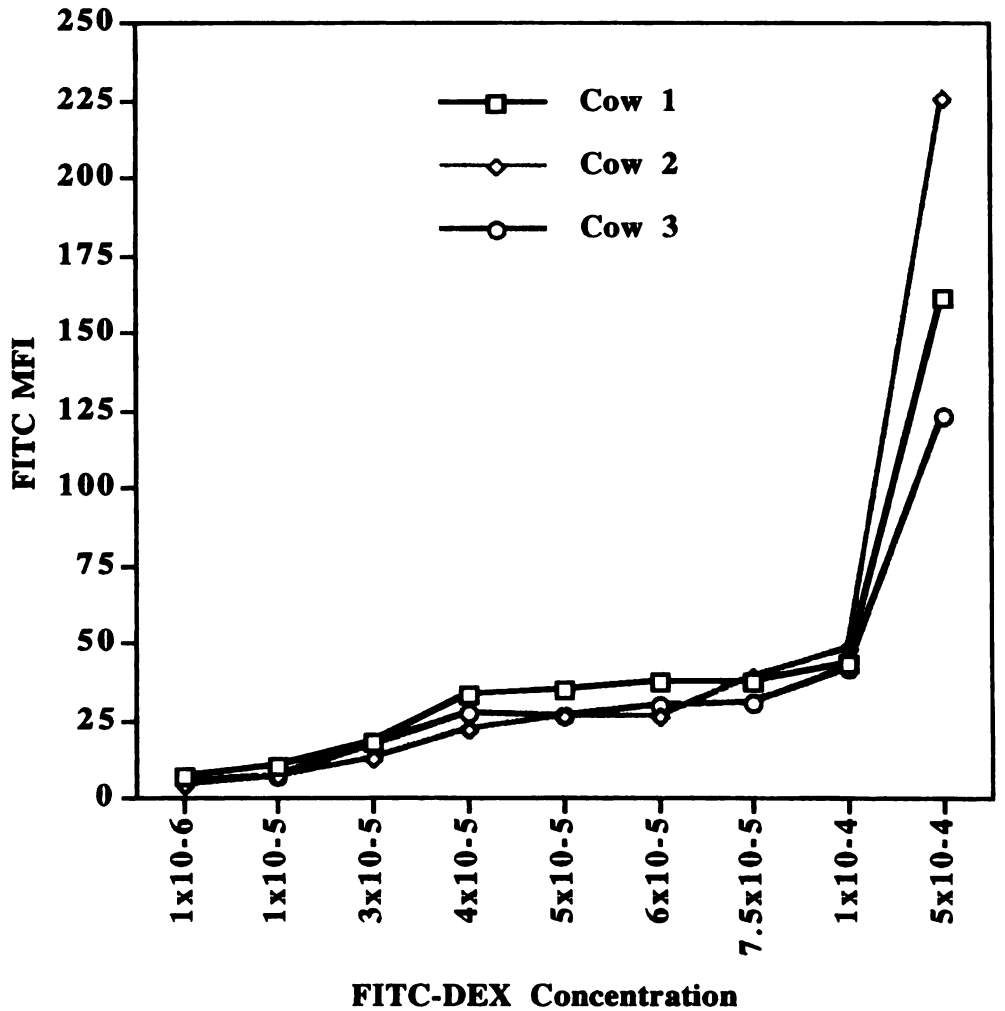
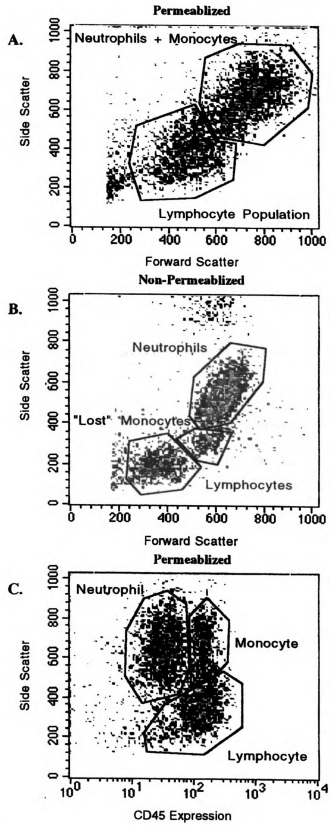


Figure 2.7 – FITC-DEX staining reached saturation within the range of 4×10^{-5} *M* and 7.5×10^{-5} *M*. Leukocytes from three cows were stained at several molar concentrations to determine concentrations of FITC-DEX that saturated bovine leukocyte GR. Two concentrations, one in saturation (5×10^{-5} *M*) and one slightly below saturation (1×10^{-5} *M*) were chosen for all future studies.

3. *Use of CD45 as the Leukocyte Differential*

Before the addition of the CD45 staining protocol to the GR staining protocol (described previously), flow cytometric analysis revealed that physical properties of permeablized leukocytes were substantially different from non-permeablized leukocytes (Figures 2.8 a and b). In essence, permeablized cells "lost" a small population of relatively large, medium granularity cells (Figure 2.8 b) that most likely corresponded to monocytes. It was speculated that the neutrophil population was significantly overlapping with the monocyte population in permeablized leukocyte preparations. This was most likely due to permeablized neutrophils plotting lower and monocytes plotting higher on the side scatter axis of density dot plots (Figure 2.8). It was therefore necessary to use a leukocyte differential to separate GR expression into the 3 major leukocyte types (neutrophils, monocytes, and lymphocytes could be established). CD45 is a popular leukocyte differential in humans (Sutherland et al., 1994; Trowbridge and Thomas, 1994). Therefore, CD45 staining was selected for use in our protocol to differentiate bovine leukocytes. As demonstrated in Figure 2.8c, CD45 is an excellent differential for bovine leukocytes. The CD45 staining protocol, as incorporated into the GR staining protocol, was described above.

Figure 2.8 – CD45 is an effective leukcoyte differential for permeablized leukocytes. (A) Flow cytometric identification of bovine leukocyte populations based only on forward-scatter (x-axis) and side-scatter (y-axis) characteristics in permeablized leukocytes gated into two regions. The larger more granular cells in the upper right region were designated as "neutrophils + monocytes". The lower region consisted of the lymphocyte population. (B) Flow cytometric identification of bovine leukocyte populations based on forward-scatter (x-axis) and side-scatter (y-axis) characteristics in non-permeablized leukocytes gated into three regions. The larger more granular cells in the upper right region were designated neutrophils, the middle region corresponded to "lost" monocytes, and the lower region consisted of the lymphocyte population. (C) Using CD45 conjugated to PE as a leukocyte differential, permeablized neutrophil, monocyte, and lymphocyte populations were distinguishable for future individual flow cytometric analyses of GR expression in individual leukocyte types.


Permeablized
A.
Neutrophils + Monocytes
Lymphocyte Population
Side Scatter
Forward Scatter
Non-Permeablized
B.
Neutrophils
"Lost" Monocytes
Lymphocytes
Side Scatter
Forward Scatter
Permeablized
C.
Neutrophil
Monocyte
Lymphocyte
Side Scatter
CD45 Expression

4. *Repeatability of MFI Values for Leukocytes Subjected to GR Staining*

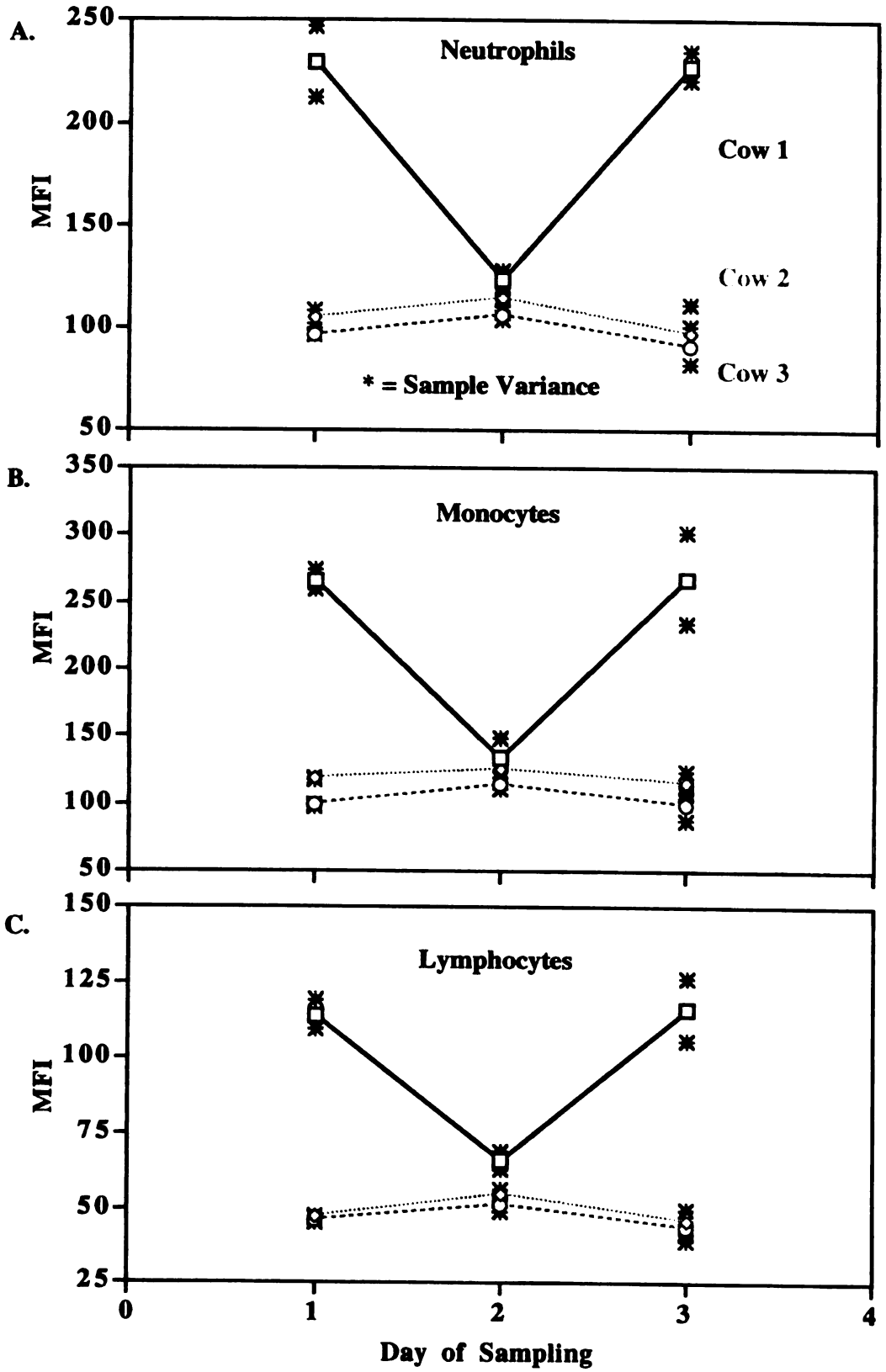
Three cows were selected for sample collections to be done on 3 consecutive days to test the repeatability of the MFI results from the dual-labeling, CD45/GR staining protocol (described above). Blood samples were drawn at 8:00 a.m. on 3 consecutive mornings and harvested leukocytes were immediately stained in duplicate with the 5 x 10^{-5} M concentration of FITC-DEX [c], and analyzed by flow cytometry as described above. GR expression (FITC-MFI) in neutrophils, monocytes, and lymphocytes were determined and Statistical Analysis Software (SAS, PROC MIXED) was then used to determine repeatability of the assay, defined as 1 minus the ratio of the variance value due to sampling errors over the total variability (sampling error, cow and cow by date). This was based on a model that included the fixed effect of date and the random effects of cow and cow by date.

Results in Table 2.2 demonstrate the degree of sampling error for GR expression (MFI). Figure 2.9 shows the FITC MFI variation within the duplicate for each cow on each sample day in neutrophils (Figure 2.9a), lymphocytes (Figure 2.9b), and monocytes. (Figure 2.9c).

Table 2.2: Repeatability of the FITC MFI results, which indicated GR expression in the 3 leukocyte types

GR Expresion in Different Leukocyte Types	**Repeatability**
Neutrophils	**96.1%**
Lymphocytes	**96.4%**
Monocytes	**93.8%**

Figure 2.9 – The repeatability of the assay was tested on duplicate samples in three cows over three consecutive days. GR expression (measured as MFI) in neutrophils (A.), monocytes (B.), and lymphocytes (C.) were determined. The blue asterisks represent the values for the duplicate samples, and the mean MFI values of the three cows are represented by the solid lines (cow 1, black; cow 2, red; cow 3, green).


A.
Neutrophils
MFI
250
200
150
100
50
Cow 1
Cow 2
Cow 3
* = Sample Variance
B.
Monocytes
MFI
350
300
250
200
150
100
50
C.
Lymphocytes
MFI
150
125
100
75
50
25
0
1
2
3
4
Day of Sampling

5. *Glucocorticoid Antagonists Compete with FITC-Dexamethasone for GR Binding*

To verify that FITC-DEX was binding with GR in bovine leukocytes, two potent GC antagonists were used in competition assays with FITC-DEX. The competition assays were performed on leukocytes left unstained for CD45, therefore, flow cytometric analysis could only be performed on "neutrophils + monocytes" and on lymphocytes (see Figure 2.10). Also, the GR staining protocol was slightly modified to accommodate the 1000-fold excess of antagonists required for successful competition assays. Therefore, the amount of FITC-DEX used was 1×10^{-6} M.

Leukocytes were prepared as described previously, but adjusted to 2×10^5 cells per well in 100 ul of PBS. Various staining and competitions were performed in 10 wells on the plate per animal. The first well was the negative control (autofluorescence; 100 ul of PBS added). The second well was for the FITC fluorochrome negative control [d] (1:100 dilution of irrelevant FITC-conjugated IgG_1 antibody added). The third well was for the unlabeled progesterone alone [k] (100 ul of 1×10^{-3} *M* progesterone). The fourth well was for the unlabeled RU486 [h] (100 ul of 1×10^{-3} *M* RU486). The fifth and sixth wells (run in duplicate) were single-color FITC-DEX positive controls [c] (100 ul of 1×10^{-6} *M* FITC-DEX). The seventh and eighth wells (run in duplicate) were designated for the competitions with progesterone. The ninth and tenth wells (run in duplicate) were for the competitions with RU486.

To start, leukocytes were fixed with 150 ul of a 1:10 dilution (in ddH_20) of FACS lysis solution that contained less than 15% formaldehyde[a] for 10 min and the solution

removed after centrifugation at 500 x *g* for 5 min. Next, the leukocytes were permeablized with 50 ul of Solution B [j] for 5 min. This was immediately slowed by the addition of 150 ul of wash solution [gg], followed by centrifugation and aspiration of supernatants. Cells were then incubated for 15 min with 100 ul of solution containing 1 $x10^{-6}$ *M* FITC-DEX [c] (wells 5 and 6), 1 x 10^{-3} *M* progesterone [k] plus 100 ul of 1 $x10^{-6}$ *M* FITC-DEX [c] (wells 7 and 8), or 1 x 10^{-3} *M* RU486 [h] plus 100 ul of 1 $x10^{-6}$ *M* FITC-DEX [c] (wells 9 and 10). All other non-competition wells also received 100 ul of incubation solution [ff]. Twenty ul of the permeabilization solution [j] was added to the incubation solution. Following the 10 min incubation, cells were washed 3 times using 200 ul of washing solution [gg] that also contained 5% permeablization solution (centrifuged at 500 x *g* for 5 min at 22^0 C). Finally, leukocytes were resuspended in 200 ul sheath fluid [f] for immediate acquisition on the FACSCalibur [w] (acquisition procedures detailed above).

Competition between FITC-DEX and progesterone or RU486 was successful in both "neutrophil + monocyte" and lymphocyte populations. Figure 2.10 shows that progesterone blocked FITC-DEX binding in "neutrophil + monocyte" by 66%, and in lymphocytes by 52%. Similar results were obtained for competitions using RU486 (Figure 2.10), though blocking with RU486 was not as effective as with progesterone. Successful blocking with both antagonists indicated that FITC-DEX was binding specifically with GR.

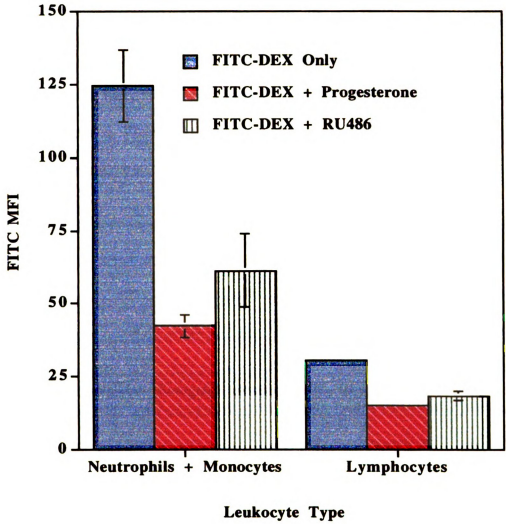


Figure 2.10 – FITC-DEX binding to GR was successfully competed by two antagonists, progesterone and RU486. FITC-DEX MFI (blue bars) was significantly decreased in both neutrophils + monocytes and lymphocytes when competed with progesterone (red bar) and RU486 (green bar).

2. *Fluorescence Microscopy to Determine FITC-Dexamethasone Location*

Fluorescence microscopy was used to visualize the location of the FITC-DEX labeling. To make slides, prepared leukocytes were first stained with propidium iodide (1:400 dilution) to stain the nuclei of the cells. After 2 washes (centrifugation at 500 x *g*, 5 min at 20^0 C) the leukocytes were stained with 5×10^{-5} *M* FITC-DEX using the protocol previously described. Figure 2.11 is a representative population of stained leukocytes, which shows cytoplasmic staining by FITC.

Note: Fluorescent photograph not reproducible for this thesis.

Figure 2.11 – Fluorescence microscopy visualization of GR in bovine blood leukocytes labeled with FITC-dexamethasone. Propidium iodide was used at a 1:400 dilution on fixed, permeablized cells to help visulaize the different nuclei morphology. The stained leukocytes demonstrate that FITC label is cytoplasmic and stains with different intensities in the different leukocyte types.

IV. Discussion

A. Dual-Color Fluorescent Labeling Protocol

The advancement of information on GR has been dependent on the technology available to study intracellular receptors. The most common method of identifying and studying GR has been with the use of isotope-labeled GR ligands, and dexamethasone had been the preferred choice due to its high affinity and specificity to GR (Norman and Litwack, 1997). More recently, fluorochrome conjugated ligands have become available. FITC-conjugated dexamethasone was the reagent used to develop a GR detection assay in bovine leukocytes. Advantages of using fluorochrome-conjugated ligands like FITC-DEX are that they are safer to use than radiolabeled ligands. The result is that GR expression can be determined quantitatively by fluorescence activated flow cytometry (Figures 2.4 and 2.5) and qualitatively by fluorescence microscopy (Figure 2.11).

Technology has taken receptor detection and quantification even further. To detect GR in human and rodent cells, polyclonal and monoclonal antibodies have been made to specific sequences of the GR protein. This allows for highly specific antibodies to be conjugated to a fluorchrome for detection of GR by flow cytometry, fluorescence microscopy, and Western blot analysis. The major obstacle to studying GR in the bovine species is that there are limited reagents available. In the future, bovine specific reagents would allow for better, more accurate detection of GR. Our lab (Weber and Burton, unpublished) is currently cloning and sequencing the bovine GR cDNA to facilitate these types of studies and to validate the results observed in the current study. However, use of polyclonal and monoclonal anti-bovine GR would be valuable, it is still of great interest

to have use of a ligand-binding assay, such as the one developed in this study, because it can yield information on receptor function that antibodies cannot. Therefore, the relative number of DEX binding sites as measured in this study by MFI can provide useful information above and beyond "identification and quantification" of receptor molecules that the use of antibodies would provide.

B. Washing Procedure

FITC-DEX at pharmacological concentrations was extremely difficult to solubilize. Therefore, the hormone was solubilized in ethanol and DMSO (5mg in 1ml ETOH + 1 ml DMSO) before the dilutions in incubation solution [ff] were made to adjust FITC-DEX to its appropriate molar concentrations. At molar concentrations above 1 x 10^{-4}, excess FITC-DEX was not washed out of leukocytes, and leukocyte physical properties were abolished. At concentrations that ranged from roughly 1 x 10^{-5} to 1 x 10^{-6} M, the use of a permeablization solution and 2 different leukocyte washing procedures could be compared. A 1 h + 3 x wash protocol was chosen to determine if the cells needed time in solution devoid of DEX to produce optimal elimination of FITC-DEX by concentration-dependent diffusion out of the cells. This was compared to a 3x wash protocol. Both washing procedures were shown to be equally effective. However, more unbound dexamethasone was washed out of permeablized cells than non-permeablized cells. The most likely reason for this is that leukocytes were fixed and permeablized before FITC-DEX staining so their plasma membranes allowed easy removal of excess probe. The washing solution contained 5% ethanol, which helped to keep FITC-DEX solubilized and binding. However, a suggestion for future research would be to include

5% of permeablization reagent such as Solution B in this solution to further facilitate removal of excess FITC-DEX during washing procedures.

C. Saturation of Leukocyte GR with FITC-Dexamethasone

In order to successfully monitor changes in bovine leukocyte GR expression in subsequent studies using periparturient cows (Chapters Three and Four), it was important to determine the molar concentrations of FITC-DEX that would saturate GR. Based on saturation curves that were repeated multiple times, the molar concentrations selected for use in this and subsequent studies were 1×10^{-5} and $5 \times 10^{-5} M$. It was interesting that the saturation curve did not permanently level off at what appeared to be GR saturation with FITC-DEX. This was difficult to explain at first; however, fluorescence microscopy revealed that the cells were lysing at very high probe concentrations and remaining cells that did not lyse became so large as to trap unbound label. The fact that some cells did not lyse at very high molar concentrations is consistent with human literature that lymphocytes subjected to suprapharmacological concentrations of DEX do not lyse (Parillo and Fauci, 1979). Therefore, this helps to explain why FITC staining could still be detected in remaining cells that had not yet lysed. Another likely possibility to explain such intense FITC fluorescence at these high molar concentrations is the probability that the hormone comes out of solution and clumps together in cells causing it to fluoresce more intensively and preventing it from being washed out by the developed protocol. In any event, concentrations in the range of 1×10^{-5} M – 5×10^{-5} M FITC-DEX worked well in the developed protocol.

D. Repeatability of Fluorescent Leukocyte GR Staining

Repeatability of the assay was performed to determine that the assay could be run on multiple animals in duplicate on different days with a low variation due to sampling error (See Table 2.2). By using the same 3 cows 3 days in a row, we had animals whose stress level would remain about the same during blood sample collections. This result was important because the design of subsequent studies to monitor GR in leukocytes of periparturient dairy cows required multiple blood sampling times (Chapters Three and Four). Results of this experiment showed that the assay was repeatable, and therefore, that it would be suitable for use in subsequent experiments.

E. FITC-Dexamethasone Binds Specifically with GR in Bovine Leukocytes

Competition assays using the glucocorticoid antagonists progesterone and RU486 demonstrated that FITC-DEX bound specifically with GR in the developed fluorescent staining and flow cytometric assay (Figure 2.10). Therefore, we were confident that conclusions in subsequent studies using periparturient dairy cows could be applied to increase basic knowledge of GR regulation in bovine leukocytes.

F. FITC-Dexamethasone Labeling is Cytoplasmic

Fluorescence microscopy of propidium iodide and FITC-DEX labeled cells appeared to be predominantly cytoplasmic. Figure 2.11 is a representative group of several leukocyte types that show the green FITC in the cytoplasm of the cells. It also indicates that there was differential expression of GR among leukocytes. The green FITC dye is more intense in the larger more granular cells and is consistent with the results observed by use of the FACSCalibur flow cytometer that monocytes and neutrophils stain brighter than lymphocytes.

V. CONCLUSIONS

Methods for GR identification and quantification continue to be developed as more information on the structure, function, and regulation of this important hormone receptor is desired. The assay developed in this study has been shown to be repeatable and specific for GR when saturating levels of FITC-DEX were used to monitor GR expression in bovine blood leukocytes. In combination with plasma membrane expression of CD45, specific leukocyte types can be monitored in future studies to determine effects of stress-related increases in blood GC concentrations on leukocyte GR expression, functional capacity, immunity, and disease susceptibility. This work could highlight that certain blood leukocytes and immune mechanisms are more susceptible to stress than others, and may target GR as a novel gene product for development of new drugs to combat certain infectious diseases in dairy cattle. In conclusion, the developed assay appears to be a reliable tool to begin to study regulation of GR in bovine leukocytes.

CHAPTER THREE

Glucocorticoid Receptor Down-Regulation in Neutrophils of Periparturient Dairy Cows

I. ABSTRACT

The objective of this study was to test effects of parturition on glucocorticoid receptor (GR) expression in neutrophils of periparturient dairy cows, and its association with blood cortisol concentrations and total blood leukocyte and neutrophil counts. Animals included 8 multiparous and 5 primiparous periparturient Holstein test cows, along with 5 multiparous and 5 primiparous mid-gestation control Holstein cows. Blood samples were collected at the following sample times relative to parturition: -28 d, -21 d, -14 d, -7 d, 0 h, 12 h, 24 h, 36 h, 48 h, 7 d, and 14 d. GR expression in neutrophils was monitored by fluorescence staining and flow cytometric acquisition and analysis, and recorded as mean fluorescence intensity (MFI) of cells. Cortisol concentrations were determined by radioimmunoassay. Total leukocytes were counted using a hemacytometer and total neutrophils determined by flow cytometric analysis of whole blood leukocytes. All variables were monitored throughout the peripartum period. The MFI values were log transformed for statistical analyses. Neutrophils from periparturient test cows showed 49 % reductions ($P < 0.0001$) in GR expression (log MFI) at calving when compared with the average GR expression before calving between –28 d to –14 d, and a 39 % reduction in GR expression when compared with neutrophils from mid-lactation, non-periparturient control cows. Depression in neutrophil GR expression started 1 wk before calving, but was most profound between 0 h and 24 h post-calving ($P < 0.0001$). There was also a significant ($P < 0.008$) difference in neutrophil GR expression profiles between primiparous and multiparous cows. Cortisol concentrations were significantly increased ($P < 0.0001$) at calving when compared to pre-calving samples, and returned to basal by 24 h post-calving. This was also true for total leukocyte counts ($P < 0.0001$). Periparturient

increases in leukocytes were due to rapid increases ($P < 0.0001$) in blood neutrophils around calving. Correlation analyses revealed significant negative relationships between neutrophil GR expression profiles and blood cortisol concentrations ($P < 0.03$), total leukocyte counts ($P < 0.05$), and neutrophil counts ($P < 0.004$). In conclusion, reduced GR expression in blood neutrophils of periparturient dairy cows is associated with elevated blood cortisol concentrations, leukocytosis, and neutrophilia around parturition. This observation implies that GR down-regulation in neutrophils may be involved in periparturient neutrophil dysregulation and, perhaps, increased mastitis susceptibility.

II. INTRODUCTION

Glucocorticoids (GC) are adrenal cortical steroid hormones that have crucial involvement in stress responses that directly affect metabolism and immune function. Their actions are mediated by intracellular glucocorticoid receptors (GR) that are located in the cytosol of target cells. Upon activation by GC, GRs translocate to the nucleus, bind to DNA in regulatory regions of target genes, and transcriptionally regulate gene expression (Bamberger, et al. 1996; DeRijk and Berkenbosch, 1994; Norman and Litwack. 1997). In situations of stress, when blood GC levels elevate beyond basal levels, GC hormones bind with high affinity to GR.

Cortisol, a natural GC, is a key hormone involved in expulsion of the fetus and initiation of lactation. Serum cortisol concentrations rise around parturition starting with a gradual increase at 3 days pre-calving followed by a dramatic increase at calving (Goff et al., 1989; Guidry et al., 1976; Smith et al, 1973). Dexamethasone (DEX), a synthetic GC, binds with high affinity and specificity to GR, and is a standard tool for studying the effects of ligand-activated GRs on stress-induced immunosuppression (Norman and Litwack, 1997). For example, lactating cows challenged with DEX experience increased shedding of bacteria, resulting in clinical mastitis (Burton and Kehrli. 1995; Lohuis et al., 1988; Wesley et al., 1989). Also, recent research using DEX-treated dairy bulls showed that some leukocyte populations are more affected by DEX than others (Burton and Kehrli. 1996; Nonnecke, et al. 1997). In one study, while DEX caused dramatic down-regulation of the adhesion molecules, CD62L (L-selectin) and CD18 on the surface of blood neutrophils, it had only modest effects on expression of these molecules on mononuclear leukocytes (Burton et al., 1995). Subsequent extension of this work to the periparturient cow model of stress showed that parturition caused a similar down-regulation of CD62L by blood neutrophils (Lee and Kehrli, 1998). This down-regulation of CD62L correlates with dysfunction of bovine neutrophil functions, especially neutrophil migration, neutrophilia, and increased susceptibility to mastitis (Cai et al., 1994; Kehrli et al., 1989a; Lee and

Kehrli, 1998; Nagahata et al., 1988). In other cells, sensitivity to GC is directly related to GR expression by the cell (Dong et al., 1988; Oakley and Cidlowski, 1993), which is usually down-regulated in response to challenge with DEX (Burnstein and Cidlowski, 1992). To our knowledge however, no studies have shown GR down-regulation in blood neutrophils following natural increases in blood cortisol.

Because neutrophils are the first line of defense against mastitis-causing organisms and depend on adequate expression of CD62L and CD18 to migrate from blood into infected mammary parenchyma,(Burton and Kehrli, 1995; Kehrli, et al. 1998), we hypothesize that cortisol-activated GRs are partially responsible for neutrophil dysfunction, which could ultimately lead to increased mastitis susceptibility of periparturient cows. To begin to test this hypothesis, we designed the current study. The objectives were to determine the effects of parturition on neutrophil cytoplasmic GR expression, and to correlate GR expression with the well documented phenomena of elevated blood cortisol concentrations, total leukocyte counts, and neutrophil counts in periparturient dairy cows.

III. MATERIALS AND METHODS

A. Cows and Blood Collection

Eight multiparous and 5 primiparous Holstein cows were used as periparturient test animals, and 5 multiparous and 5 primiparous mid-gestation Holsteins as non-periparturient control animals. Blood samples for flow cytometric analysis of neutrophil GR expression and leukocyte counting were collected by tail veinupuncture into Vacutainer brand blood collection tubes containing 1.0 ml of acid citrate dextrose anticoagulant [p, q] using 20*g* 2.5 cm multi-sample needles [o]. Samples for serum harvesting (cortisol assay [b]) were collected into Vacutainer tubes that contained no anticoagulant [r]. The schedule for sampling included 11 samples relative to parturition (-28 d, -21 d, -14 d, -7 d, 0 h, 12 h, 24 h, 36 h, 48 h, 7 d, and 14 d). Pre- and post-partum samples were collected at 0800

h, while the intense sampling around calving occurred at precise hours relative to the 0 h (calving) sample.

B. Leukocyte Counting and Standardization of Cell Numbers for GR Staining

Total leukocytes were counted to standardize the number of cells (5 x 10^6 cells) added per well of 96-welled U-bottom microtiter plates [t, u] for fluorescent staining, as well as for future use in determining neutrophil counts. Red blood cells from 5 ml of whole blood were lysed for 1.5 min using 10 ml cold hypotonic lysing solution [cc] (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, pH 7.3). This was followed by addition of 20 ml cold hypertonic restoring solution [dd] (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, 0.43 M NaCl, pH 7.3). Remaining leukocytes were centrifuged [y, used for all centrifugation] at 1000 x *g* for 5 min at 22^0 C and supernatants aspirated. The cell pellet was washed twice in 2 ml cold PBS [ee] and centrifuged at 800 x *g* for 5 min at 22^0 C. Leukocytes were counted on a hemacytometer [z] and adjusted to 5 x 10^6 cells in PBS per well, and subjected to fluorescence staining (below).

C. Fluorescence Staining of Neutrophil GR

1. General Procedures

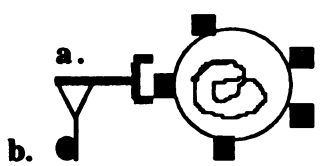
Prepared leukocytes (above) were differentially stained on their plasma membranes and then cytoplasmic GRs were labeled. CD45, a plasma membrane protein, was immunolabeled with phycoerythrin (PE) as a leukocyte differential (Sutherland et al, 1994; Trowbridge and Thomas, 1994), and fluorescein isothiocyanate- (FITC-) conjugated DEX was used to label cytoplasmic GR. Dexamethasone is commonly used to identify GR in target tissues (Rupprect et al., 1993). The final result was a dual-color staining protocol (Figure 3.1). For each animal sampled, seven wells were used for flow cytometric acquisition. The first well was a negative control (autofluorescence of neutrophils; 100 µl of PBS), the second well was the FITC fluorochrome negative control [d] (1:100 dilution

of irrelevant FITC-conjugated IgG_1 antibody added); the third well was the PE fluorochrome negative control [g] (1:100 dilution of irrelevant PE-conjugated IgG_{2a} antibody added); the fourth well was a single-color PE-stained CD45 control [i, e] (100 µl of 5 µl/ml primary anti-CD45 antibody added, followed by 100 µl of a 1:400 dilution of PE-conjugated anti-IgG_{2a} secondary antibody); the fifth well was a single-color FITC-stained GR control [c] (100 µl of $1x10^{-5}$ M FITC-DEX); wells 6 and 7 were designated for the final two-color staining including both CD45 (PE) and GR (FITC-DEX). Preliminary saturation curves consistently demonstrated that $1x10^{-5}$ M and $5x10^{-5}$ M FITC-dexamethasone caused GR saturation, so these were the concentrations selected for wells 6 and 7, respectively.

Step 1. CD45 Staining as a Leukocyte Differential Using:

a. Primary anti-bovine CD45 antibody

b. Secondary Mouse anti-IgG2a antibody



Step 2. Cytoplasmic Neutrophil GR Staining using:

c. FITC-conjugated dexamethasone

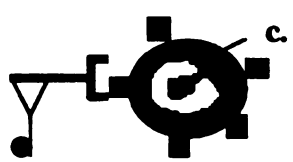


Figure 3.1 - Model of two-color fluorescence staining of plasma membrane CD45 and cytoplasmic glucocorticoid receptors.

2. CD45 Staining

The CD45 staining protocol (Step 1 of Figure 3.1) started with a 30 min incubation at 4^0 C in the dark with the anti-CD45 antibody [i] (wells 4, 6, 7; remaining wells received 100 µl of PBS). The reaction was slowed by the addition of 100 µl cold PBS, plates centrifuged at 500 x *g* for 5 min at 22^0 C, and supernatants aspirated. Cells were resuspended in secondary antibody [e] (wells 4, 6, and 7), negative isotype control antibody [g] (well 3), or PBS (wells 1 and 2) for 30 minutes at 4^0 C in the dark. After washing the cells in 200 µl of PBS, plates were centrifuged and supernatants aspirated as above.

3. GR Staining

Cells were then fixed with 150 µl of a 1:10 dilution (in ddH_20) of FACS Lysis solution [a] for 10 min and the solution removed after centrifugation at 500 x *g*. Next, the cells were permeablized with 50 µl Solution B [j] for 15 min. This was immediately slowed by the addition of 150 µl wash solution [gg] [10 mM Tris (pH 7.6) containing 0.1% Tween 20 and 5% ethanol], followed by centrifugation and aspiration of supernatants. Cells were then incubated with 100 µl of either 1×10^{-5} M (wells 5 and 6) or 5×10^{-5} M FITC-DEX [c] (well 7), or the FITC negative control [d] (well 2), and remaining wells received 100 µl of incubation solution [10 mM Tris (pH 7.6) containing 0.1% Tween 20 and 1% BSA]. The cells were incubated for 10 min at 22^0 C. Following incubation cells were washed 3 times in washing solution [gg], centrifuged at 500 x *g* for 5 min at 22^0 C and resuspended in 200 µl sheath fluid [f] for immediate acquisition on a fluorescence activated flow cytometer (below). Using this protocol for GR staining (Figure 3.1, Step 2), we were able to determine by fluorescence microscopy [aa] that FITC-DEX fluorescence was located in the cytoplasm of bovine neutrophils (Figure 3.2). Furthermore, 100 fold excess of unlabeled progesterone [k], a natural GR antagonist (Gagne et al., 1986; Rupprect et al., 1993), was able to compete with FITC-DEX for GR

binding sites, causing greater than 34 % reductions in the intensity of the FITC signal (data not shown).

Figure 3.2 – Fluorescence microscopy visualization of GR in a bovine blood neutrophil labeled with FITC-dexamethasone. Propidium iodide was used at a 1:400 dilution on fixed, permeablized cells to help visualize the segmented nuclei of neutrophils (red staining) and FITC-dexamethasone was used at 1 x 10^{-5} M to probe for GR (green staining). A representative neutrophil is shown and demonstrates that the FITC label is exclusively cytoplasmic.

Note: Fluorescent photograph not reproducible for this thesis.

D. Flow Cytometric Acquisition of Neutrophil GR Expression Data

A FACSCalibur [w] fluorescence activated flow cytometer and the acquisition package of CellQuest software [v] were utilized to acquire neutrophil GR expression data. Unstained cells in well 1 were used to check the forward scatter (FSC = size characteristics) and side scatter (SSC = granularity characteristics) parameters of the leukocytes (Figure 3.3 a). These characteristics allowed individual animal variation in leukocyte physical properties to be adjusted in FSC-SSC density plots. Based on one-color stained cells in wells 5 and 4, FL-1 (FITC) and FL-2 (PE) detectors were adjusted in preliminary experiments for optimal demonstration of green and red fluorescence, respectively, and were used to optimize compensation for the two-color analyses of cells in

wells 6 and 7. FL-1, FL-2, and compensation settings were not altered during the actual trial. Cells from wells 2 (FITC control) and 3 (PE control) were used to set background fluorescence of the FITC and PE fluorochromes, respectively. Finally, cells in wells 6 and 7 were collected as the dual-colored CD45/GR data sets used for subsequent hypothesis testing. Five thousand events were acquired from each of these wells for further flow cytometric and statistical analyses (see below).

E. Flow Cytometric Analysis of Neutrophil GR Expression Data

The analysis package of CellQuest software [v] was used for determining neutrophil GR expression based on FITC fluorescence (mean fluorescence intensity, or MFI) of dual-gated, CD45-differentiated leukocytes (Figure 3.3). Neutrophils were identified using both physical (Figure 3.3a) and CD45 expression characteristics (Figure 3.3b). FL-1 fluorescence histograms were then plotted for the double-gated leukocytes identified as neutrophils and MFI of those cells used to indicate neutrophil GR expression (Figure 3.4). A shift to the left of the histogram peak represents decreased expression of neutrophil GR, while a shift to the right indicates increased GR expression. Therefore, shifts in MFI of the FL-1 peak from CD45-differentiated neutrophils were used to assess effects of parturition on neutrophil GR expression.

F. Neutrophil Counts

Neutrophil counts (# cells/ml) were determined by multiplying total leukocyte counts (# cells/ml) by the percentage (%) of differentiated neutrophils determined by flow cytometry (using one-color CD45 stained cells in well 4) (Figure 3.3b).

G. Blood Cortisol Concentrations

Cortisol concentrations were determined by radioimmunoassay (RIA). Sera were harvested from each blood sample following clotting and centrifugation of blood tubes at 1000 x *g*, and were stored at -20^0 C until use in the RIA [b]. All sera were assayed in duplicate on 1 day and data recorded as cpm using a gamma counter [x]. The cpm were

then converted to ug/dL of cortisol based on a standard curve of cpm by concentration. The intraassay coefficient of variation was less than 10%.

H. Statistical Analyses

Statistical Analysis Software (SAS) was used to analyze data sets by repeated measures analysis (SAS, 1996). The mixed model applied to all data sets included a random cow effect and fixed effects of parity, time (relative to calving), treatment group (control vs test), calendar week of sampling (accounted for assay variation during the study), and a fixed time-by-parity interaction (Model 1). Due to the design of the current study, the other two-way interactions were confounded and were not analyzed. Upon initial examination of the distribution of the GR expression data, a log transformation was applied. Time and time-by-parity least squares means (LSMs) were reported for neutrophil GR expression (log MFI), cortisol (ug/dL), and leukocyte and neutrophil counts (#cells/ml). The correlation procedure of SAS (SAS, 1990) was used to determine associations between neutrophil GR expression and blood cortisol concentrations, leukocyte counts, and neutrophil counts.

Model [1]

$$Y_{ijklmn} = m + A_i + P_j + T_k + G_l + E_m + T \times P_{kj} + error_n$$

Where: Y_{ijklmn} = observation on the i^{th} animal in her j^{th} parity at the k^{th} sample time relative to calving, for the l^{th} treatment group and the m^{th} week of sampling.

A_i = random cow effect (i = 1, 2, 3, ... 23)
P_j = fixed parity effect (j = primiparous, multiparous)
T_k = fixed time effect relative to calving (1, 2, 3, ... 11)
G_l = fixed treatment group (control, sample)
E_m = fixed calendar week of sampling effect (1, 2, 3, ... 10)
$T \times P_{kj}$ = fixed time by parity interaction effect
$error_n$ = random error term

IV. RESULTS

A. Neutrophil GR Expression

Flow cytometric analysis of neutrophils showed that parturition induced down-regulation of GR expression (Figure 3.4; 1 x10 $^{-5}$ M FITC-DEX) when FITC- DEX was used in the assay at 1 x10 $^{-5}$ M and 5 x10 $^{-5}$ M. Because there was a highly significant correlation between the 2 concentrations (r = .81, $P < 0.0001$), indicating that neutrophil GRs were stained at saturation, LSMs of GR expression are shown for only the data acquired at 1 x10 $^{-5}$ M FITC-DEX. Analysis of variance of neutrophil GR expression revealed significant effects of time, calendar week, and the parity-by-time interaction (Table 3.1). The significance of the time effect was that it was the actual parturition effect that our hypothesis was testing. As demonstrated by the time LSMs in Figure 3.5, parturition caused a significant reduction in FITC-DEX staining ($P < 0.05$) in blood neutrophils which started approximately one week prior to calving. The figures displaying time relative to parturition on the x-axis are reported in days relative to parturition to provide accuracy, therefore, -1wk equals - 7 d in Figure 3.5. By calving, neutrophils from test animals displayed dramatically reduced GR expression ($P < 0.0001$; Figure 3.5), which persisted to 24 h and remained significantly lower ($P < 0.05$) than control GR values throughout the remaining 2 wk post-calving. This is well demonstrated for a representative test cow in Figure 3.4, which shows a dramatic shift to the left of the FITC histogram peak at 0 h (calving) relative to the –3 wk pre-calving peak, and incomplete recovery of GR expression at 1 wk post-calving. MFI of all periparturient test animals at calving demonstrated a shift to the left of pre-calving histogram peaks (represented for one animal in Figure 3.4). A significant parity-by-time interaction ($P < 0.008$; Table 3.1) was also observed in this GR expression data set. Parity-by-time LSMs in Table 3.2 show that primiparous animals had larger reductions but quicker recovery of neutrophil GR expression than multiparous animals.

B. Blood Cortisol Concentrations

Analysis of variance showed that the main effect of time relative to parturition was significant ($P < 0.0001$; Table 3.1) for blood cortisol concentrations. Time LSMs for cortisol were increased nearly 7-fold at calving compared to control and pre-calving means (Figure 3.6). However, blood cortisol returned to control values by 24 h post calving.

C. Total Leukocyte and Neutrophil Counts

Analysis of variance of total leukocyte and neutrophil count data sets showed that the main effect of time relative to parturition was highly significant ($P < 0.0001$; Table 3.1). Time LSMs in Figure 3.7 demonstrate that parturition caused a significant ($P < 0.001$) elevation in leukocyte counts that persisted until 12 h post-calving. This leukocytosis was explained by a dramatic neutrophilia ($P < 0.0001$; Figure 3.7b), that mirrored kinetics of leukocyte counts (Figure 3.7a).

D. Correlation Analyses Between Data Sets

Table 3.3 displays the correlation matrix for neutrophil GR expression, blood cortisol concentration, and total leukocyte and neutrophil counts. Blood cortisol concentrations were highly positively correlated with total leukocyte and neutrophil counts ($P < 0.001$) and negatively correlated with neutrophil GR expression ($P < 0.05$). Neutrophil GR expression was negatively correlated with total leukocyte ($P < 0.05$) and neutrophil counts ($P < 0.01$). Total leukocyte and neutrophil counts were highly positively correlated ($P < 0.001$).

V. DISCUSSION

During the peripartum period of 3 wk prior to calving through 3 wk post calving, dairy cows become immunosuppressed (Dettileux, et al. 1995). Associated with periparturient immunosuppression are higher rates of new intramammary infections (Oliver and Sordillo, 1988). This has led to unacceptably high occurrences of mastitis in the first 30-60 days of lactation (National Mastitis Council, 1996). Our hypothesis was that GR, a

high affinity receptor for cortisol, is involved in periparturient immunosuppression related to impaired expression of adhesion molecules (Lee and Kehrli, 1998) and functional capacity of blood neutrophils (Cai et al., 1994). Rapid recruitment of neutrophils into newly infected mammary parenchyma is the key defense against mastitis causing pathogens in ruminants (Miller et al., 1994).

The observed decrease in GR expression of neutrophils (Figures 3.4 and 3.5) imply that cortisol-activated GR translocates into the nucleus of bovine neutrophils around the time of parturition, possibly binding to target genes to regulate their transcription. This has been demonstrated using artificial systems of GR activation, such as transformed cell lines exposed to DEX (Burnstein and Cidlowski, 1992; Dong et al., 1988; Oakley and Cidlowski, 1993). Indeed, altered gene transcription mediated by GC-bound GR could explain reduced expression of certain plasma membrane proteins on neutrophils, such as CD62L that is severely down-regulated in cattle treated with DEX (Burton and Kehrli, 1995; Burton and Kehrli, 1996; Burton et al., 1995), and cows at parturition (Lee and Kehrli, 1998). This could be the cause of leukocytosis and neutrophilia (Figure 3.7) that has been shown in other studies of periparturient dairy cows (Detilleux, et al. 1994; Guidry, et al. 1976) and, if so, strongly implicates cortisol activation of neutrophil GR as a mechanism that causes neutrophilia. This notion is supported by our observation that GR down-regulation and neutrophilia are highly correlated, as are cortisol concentrations and blood neutrophil counts. Therefore, our study confirms and extends results of others (Goff et al., 1989; Guidry et al., 1976; Smith et al., 1973) by suggesting a link between cortisol-induced neutrophil GR down-regulation (Figure 3.5) and neutrophil dysfunctions observed by others in periparturient dairy cows (Cai et al., 1994; Kehrli et al., 1989; Lee and Kehrli, 1998; Nagahata et al., 1988). Furthermore, GR expression results of this study are the first that show down-regulation of GR in blood neutrophils of healthy animals undergoing a natural stress that includes significant and sustained increases in blood cortisol.

Parturition-induced reductions in neutrophil GR expression could be explained in one of three ways. First, our fluorescence staining and flow cytometric assay detects cytoplasmic GR (Figure 3.2), so if increased blood cortisol did cause GR translocation to the nucleus, these GR would be undetectable in our assay. Secondly, our assay detects DEX-binding, not the presence of GR protein per se, so it is possible that the GC binding site was altered to exclude FITC-DEX binding without altering the actual number of receptors. The definitive answer to this possibility awaits development of bovine specific anti-GR antibodies. Work in other species has shown that decreased GC binding following GC challenge is caused by a reduction in GR numbers (Burnstein and Cidlowski, 1992; Dong et al., 1988; Oakley and Cidlowski, 1993). Another possibility to explain GR down-regulation in this study is that bovine GR represses GR gene expression. Substantial evidence from human literature indicates that GR is autoregulatory (Burnstein and Cidlowski, 1992; Dong et al., 1988; Oakley and Cidlowski, 1993), and that GC-mediated GR down-regulation is caused in most cells types by reduced GR mRNA due to reduced rates of transcription and reduced stability of the GR mRNA (Burnstein and Cidlowski, 1992; Dong et al., 1988; Oakley and Cidlowski, 1993). If the same were true of bovine neutrophil GR, one would expect reduced GR mRNA and protein in neutrophils of periparturient cows. We have taken steps to demonstrate GR protein down-regulation (Figures 3.4 and 3.5) in response to elevated cortisol (Figure 3.6) and are currently investigating effects of parturition on neutrophil GR mRNA abundance. All possibilities for GR down-regulation could occur in neutrophils and imply that GR is involved not only in the regulation of critical molecules necessary for effective neutrophil-mediated immunity in periparturient dairy cows (eg. CD62L), but also in its own regulation.

Finally, it was intriguing that multiparous animals of our study had prolonged GR down-regulation relative to primiparous animals (Table 3.2). Multiparous cows are more prone to health disorders, such as mastitis and ketosis, than primiparous heifers (Curtis et al., 1985; Grohn et al., 1995). It is noteworthy, therefore, that primiparous animals in this

study did not develop any metabolic disorders or clinical mastitis post-calving, while over half of the multiparous cows had some combination of these disorders. For example, 2 multiparous cows received surgery for displaced abomasums, 2 were treated for severe ketosis and one for metritis. The other multiparous cows appeared healthy. Low animal numbers in this study precluded statistical analysis of health effects on neutrophil GR. Nonetheless, the severity and persistency of reduced neutrophil GR expression among multiparous cows of this study suggests that homeostasis of these animals remained stressed even though blood cortisol concentrations returned to normal. The multiparous animals were trying to overcome stress of parturition and subsequent metabolic disorders, which could have resulted in a longer recovery as measured by reduced neutrophil GR expression. It is also possible that neutrophils of multiparous cows are more sensitive to cortisol than cells of younger animals as is implied by their higher GR expression pre-calving (Table 3.2). If so, this could explain both sustained neutrophil GR down-regulation observed in this study and, as observed by others, increased mastitis susceptibility in multiparous versus primiparous periparturient cows (Curtis et al., 1985; Grohn et al., 1995). In either scenario, results of the current study suggest that neutrophil GR expression may be a better biological indicator of stress and immunosuppression than blood cortisol and may even explain the occurrence and severity of bacterially-induced disease susceptibility following stress (Burton and Kehrli, 1995; Kehrli et al., 1997; Lohuis et al., 1988; Wesley et al., 1989).

In conclusion, this study demonstrated that elevated maternal blood cortisol concentrations are associated with both neutrophilia and down-regulation of cytoplasmic GR in blood neutrophils of periparturient dairy cows. The observed GC-induced GR down-regulation has implications for stress-induced immunosuppression and increased mastitis susceptibility, especially in multiparous cows around parturition.

Chapter Three

Tables and Figures

Table 3.1 - Results of the Analysis of Variance Using Model [1]

	Neutrophil GR Expression		Cortisol	Leukocyte Counts	
Model Effect	$1x10^{-5}$ M FITC-DEX	$5x10^{-5}$ M FITC-DEX		Total	Neutrophil
Time	0.0002	0.0071	0.0001	0.0001	0.0001
Parity	0.0644	0.6928	0.1571	0.7110	0.4728
Env. Period	0.0001	0.0001	0.7174	0.0409	0.7555
Parity x Time	0.0079	0.0143	0.1435	0.1842	0.1092

Table 3.2 – Parity x Time LSMs of log10-transformed Neutrophil GR Expression (MFI) Data

Time Relative to Calving	Multiparous Control Mean	Multiparous Parturient Means	Primiparous Control Mean	Primiparous Parturient Means
	4.63		4.56	
-28 d		5.26[a]		4.19[b]
-21 d		4.87		4.76
-14 d		4.72		4.78
-7 d		4.81		4.09
0 h		4.37[a]		3.82[*b]
12 h		4.22[*]		4.05[*]
24 h		4.38		4.11
36 h		4.17[*]		4.65
48 h		4.48		4.50
7 d		4.37		4.37
14 d		4.58		4.30

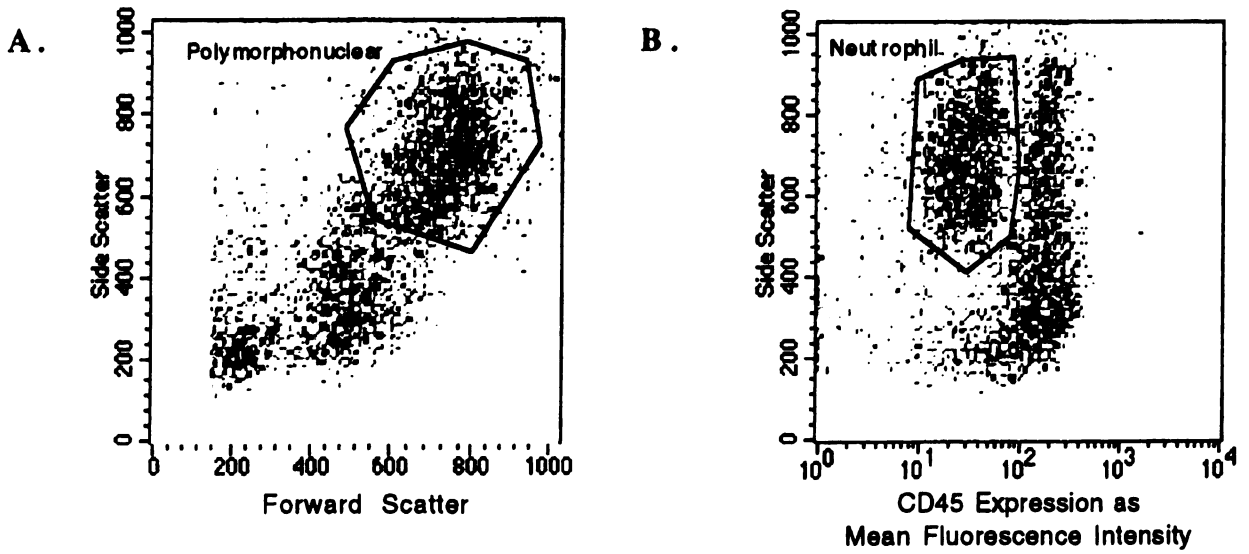
* = differences ($P < 0.05$) from respective parity control mean
a,b = multiparous cows different ($P < 0.05$) from primiparous heifers when compared at the same time relative to calving

Table 3.3 - Correlation Statistics

	Neutrophil GR Expression	Cortisol	Total Leukocyte	Total Neutrophil
Neutrophil GR Expression	1.00			
Cortisol	-0.2*	1.00		
Total Leukocyte	-0.17*	0.58***	1.00	
Total Neutrophil	-0.26**	0.62***	0.86***	1.00

* = $P < 0.05$
** = $P < 0.001$
*** = $P < 0.0001$

Figure 3.3 – Flow cytometric identification of bovine neutrophils based on (A) forward - side scatter properties, and (B) CD45 expression – side scatter properties. Cells that fell within the polymorphonuclear (A) and neutrophil (B) gates were designated as neutrophils, and were subsequently assessed for GR expression.



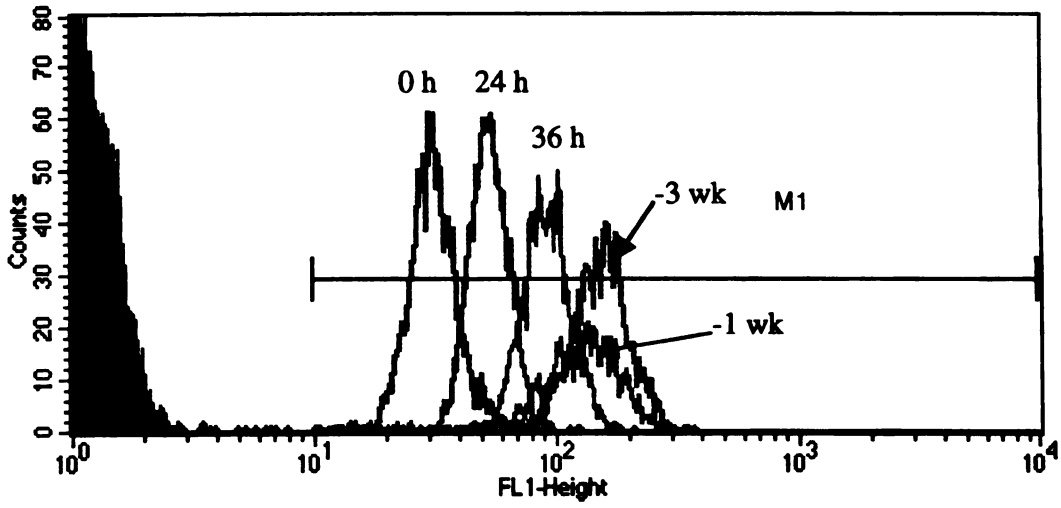


Figure 3.4 – Bovine neutrophil GR down-regulation at parturition as determined by the mean fluorescence intensity (MFI) of FITC. FITC-DEX was used to label GR in blood neutrophils of periparturient cows. Shown are representative FITC histogram peaks for one cow for neutrophils sampled 3 wk pre-calving (-3 wk; dark green), at calving (0 h; dark blue), 24 h post-calving (24 h; red), 36 h post-calving (36 h; orange), and 1 wk post-calving (1 wk; cyan). Background fluorescence of the FITC negative control is shown as a solid green peak.

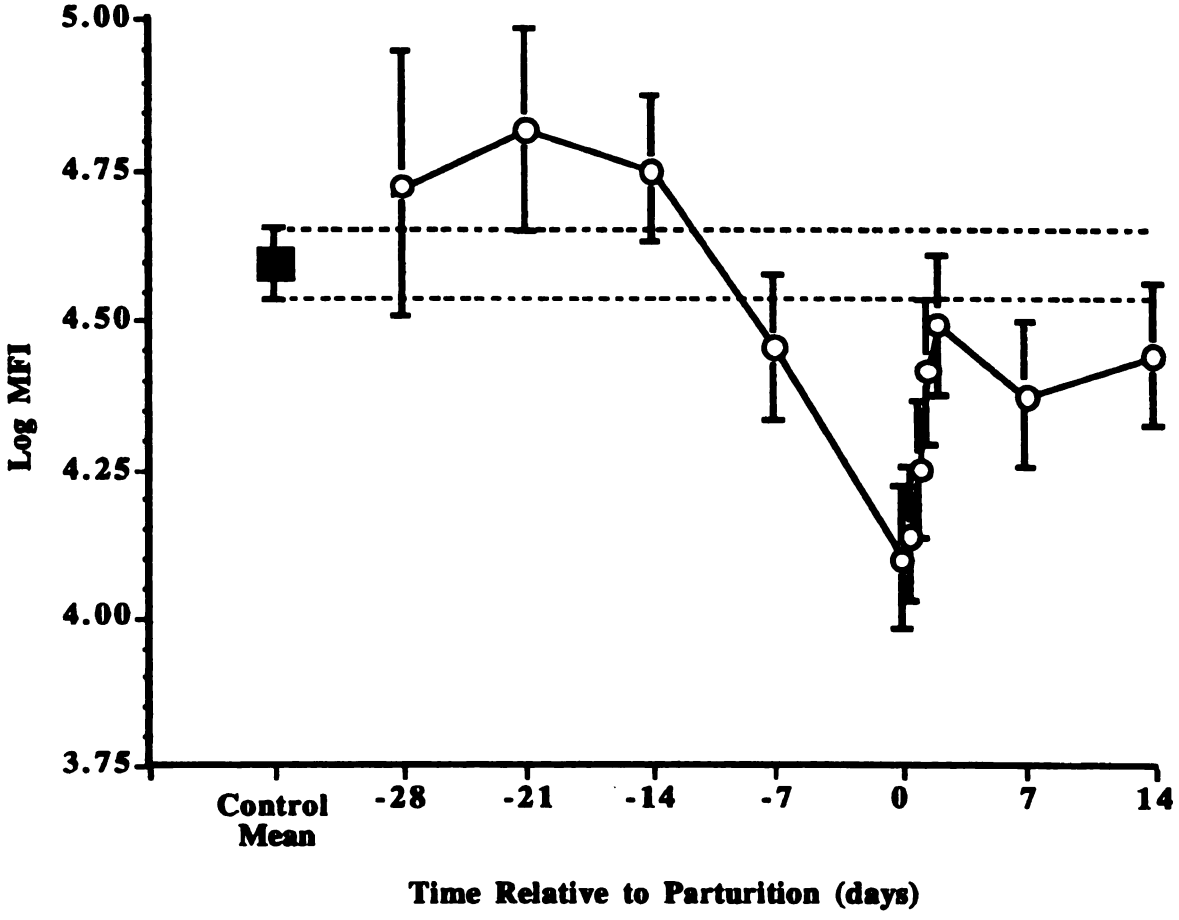


Figure 3.5 – Time LSMs (±SEM) of log-transformed neutrophil GR expression measured as mean fluorescence intensity (MFI) of FITC-DEX labeled GR. Time was relative to parturition at time 0. Solid square and connected dashed lines are the overall control mean ± SEM. Open circles and connecting solid line are data for the periparturient test cows. Parturition caused a 39-49 % decrease in bovine neutrophil GR expression (log of MFI), depending on whether comparisons were with the control mean or the day –28 to –14 pre-calving mean, respectively.

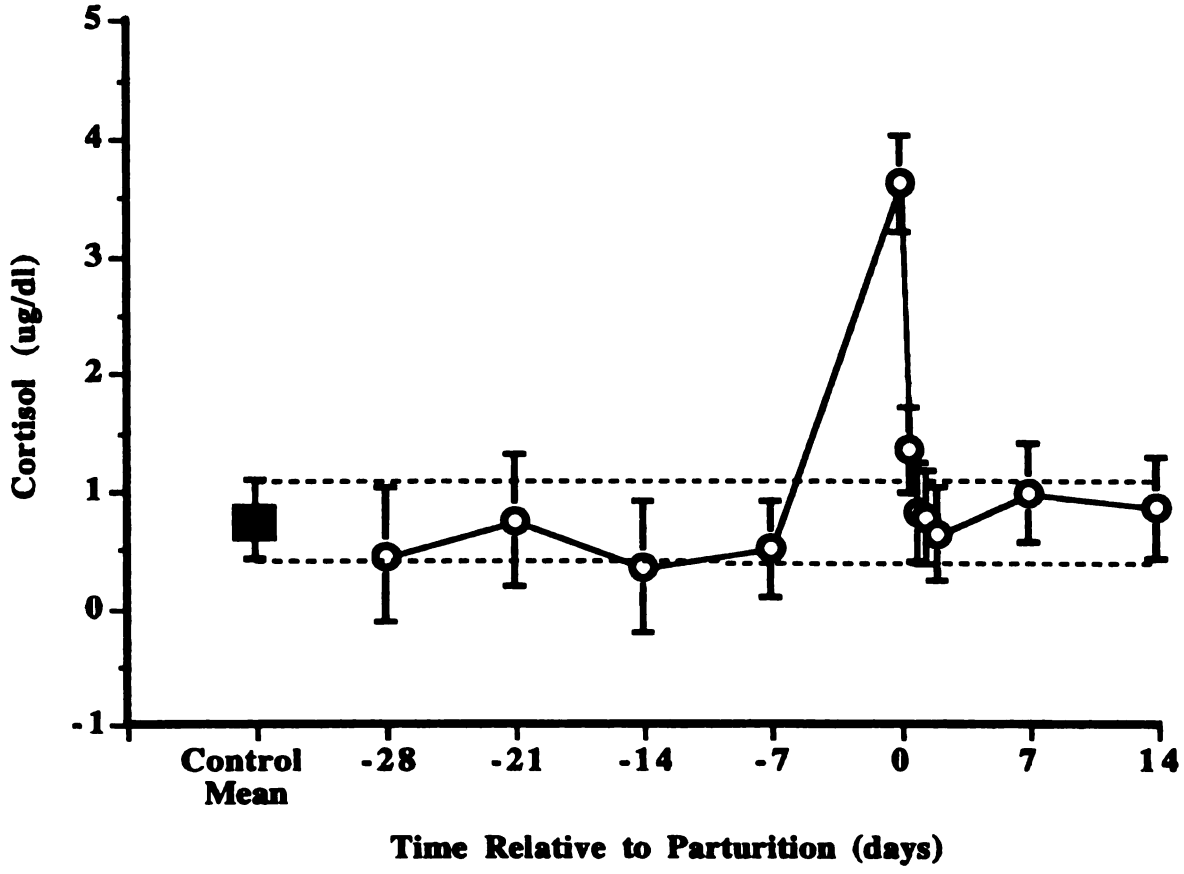


Figure 3.6 – Time LSMs (±SEM) of blood cortisol concentrations. Time was relative to parturition at time 0. Solid square and connected dashed lines are the overall control mean ±SEM. Open circles and connecting solid line are data for the periparturient test cows. Parturition caused an approximate 7-fold increase in blood cortisol concentration.

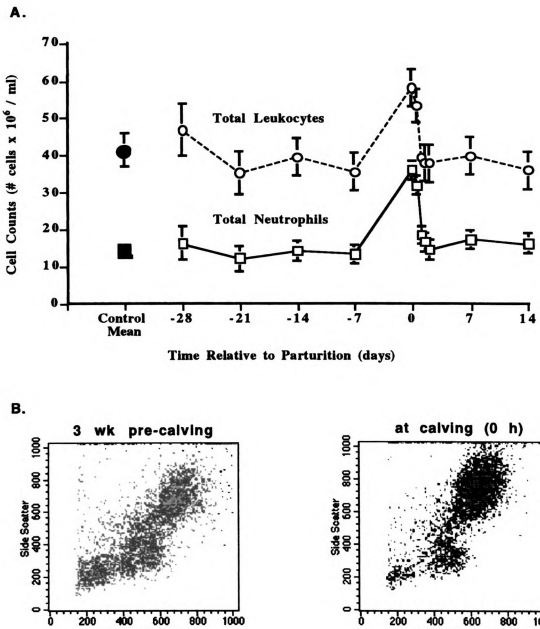


Figure 3.7 – (A) Time LSMs (±SEM) of total leukocyte and neutrophil counts (#cells/ml), and (B) representative density dot plots for one test cow showing a large increase in the neutrophil fraction of blood leukocytes at calving. In (A), solid circle and square are the overall control means ±SEM, and open circles and squares with connecting dotted and solid lines (respectively) are data for the periparturient test cows. Calving caused an ~50 % increase in blood leukocytes which was accounted for by the pending neutrophilia.

CHAPTER FOUR

Glucocorticoid Receptor Expression Profiles in Mononuclear Leukocyte Populations of Periparturient Holstein Cows

I. ABSTRACT

Cortisol-activated glucocorticoid receptors (GR) modulate cellular responses to stress by translocating from the cytosol to the nucleus and enhancing or repressing the transcription of target genes. The functional capacity of mononuclear leukocytes is inhibited in parturient dairy cows at a time when blood cortisol concentrations are high. Because GR is autoregulatory in many cell types, the hypothesis of the current study was that GR expression by mononuclear leukocytes is altered around parturition in association with elevated blood cortisol levels. If true, GR could be involved in suppressed functions of mononuclear leukocytes in parturient cows. The objectives of this study were to determine effects of parturition on lymphocyte and monocyte GR expression and to correlate expression with serum cortisol concentrations. Objectives were achieved by using fluorescence staining and flow cytometric analyses to monitor GR in peripheral blood mononuclear leukocytes collected multiple times from 13 periparturient test cows (8 multi- and 5 primiparous) and 10 midgestation control cows (5 multi- and 5 primiparous). Serum cortisol concentrations were determined by radioimmunoassay. Based on intensity of the fluorescent GR stain, parturition caused 42 and 47% reductions ($P < 0.001$) in lymphocyte and monocyte GR expression, respectively, compared to mean expression in cells from control cows. When mean prepartum values were compared with nadir values at parturition in the test cows, GR expression was reduced by 67% in lymphocytes and by 54% in monocytes ($P < 0.001$). Mononuclear cell expression of GR was negatively correlated with serum cortisol concentrations. Results suggest that GR is down regulated

in bovine mononuclear leukocytes in association with increased adrenal secretion of cortisol at calving. It is possible that GR down-regulation is also associated with altered phenotype or function (or both) of these lymphocytes and monocytes. This possibility should be substantiated because it could explain increased disease susceptibility in periparturient dairy cows.

II. INTRODUCTION

Dairy cows are susceptible to immunosuppression and mastitis during the peripartum period, a time when blood concentrations of the stress hormone, cortisol, are high (Goff et al., 1989; Guidry et al., 1976; Smith et al., 1973). Cortisol is an endogenous glucocorticoid (GC) that delivers its hormonal message to cells via cytoplasmic glucocorticoid receptors (GR). When bound to GC, GR become activated, translocate into the nucleus, bind to regulatory regions in DNA of glucocorticoid responsive genes, and either repress or enhance expression of those genes (Bamberger et al., 1996; Burnstein and Cidlowski, 1992; DeRijk and Berkenbosch, 1994; Norman and Litwack, 1997; Oakley and Cidlowski, 1993). Therefore, glucocorticoids are critical contributors to altered phenotypes and functions of target cells during stress.

Recent research from our laboratory demonstrated that GR expression is down-regulated in blood neutrophils of periparturient dairy cows in conjunction with elevated blood cortisol concentrations (Preisler et al., 1999). Homologous GR down-regulation has been well documented in a variety of GC responsive systems (De Rijk and Berkenbosch, 1994), and presumably provides a short-loop feedback mechanism to protect cells against prolonged exposure to GC (Oakley and Cidlowski,1993). Along with homologous GR down-regulation, GC responsive systems also alter expression of other GC responsive genes when GR is activated, thus changing the phenotype and function of the target cells. Down-regulation of the CD62L adhesion molecule is an example of a phenotypic change in response to GC that has been documented in blood neutrophils from both periparturient

dairy cows (Lee and Kehrli, 1998) and dairy cattle injected with the potent synthetic GC, dexamethasone (Burton et al., 1995). Glucocorticoid-induced CD62L down-regulation is associated with dysfunctional neutrophil trafficking that results in neutrophilia and increased mastitis susceptibility (Burton and Kehrli, 1995). Indeed, mastitis susceptibility and neutrophil dysfunctions related to altered trafficking of these leukocytes are well documented for both periparturient and dexamethasone-treated dairy cows (Cai et al., 1994; Kehrli et al., 1998; Lohuis et al., 1988; Nagahata et al., 1988; Oliver and Sordillo, 1988; Roth and Kaeberle, 1982; Wesley et al., 1989). Therefore, GR is strongly implicated in neutrophil dysfunctions and mastitis susceptibility of GC-challenged dairy cows.

Mononuclear leukocyte phenotypes and functions of periparturient cows (Detilleux et al., 1995; Kehrli et al., 1989a; Kehrli et al., 1989b; Mallard et al., 1998; Sordillo et al., 1991) and dexamethasone-treated cattle (Burton and Kehrli, 1996; Nonnecke et al., 1997; Roth and Kaeberle, 1982) are also altered. For example, when leukocytes are collected from cows with elevated blood GC, mononuclear cell expression of MHC I and II antigens is down-regulated. Also, trafficking patterns of monocytes and γT cells are altered in vivo and in vitro proliferation, cytokine, and antibody production in response to mitogens and antigens are inhibited. Each of these cellular phenotypic changes may jeopardize adaptive immunity and suggest that GR could be involved in suppressed immunity and disease susceptibility of GC-challenged cows.

The hypothesis of the present study was that parturition affects bovine mononuclear leukocyte expression of GR and that this is associated with elevated blood cortisol concentrations at calving. The objectives were to monitor GR expression in mononuclear cells throughout the peripartum period and to correlate GR expression with blood cortisol concentration and circulating numbers of lymphocytes and monocytes. Given that homologous down-regulation of GR is caused by GR activation, demonstration of altered GR expression in mononuclear cells in association with elevated blood cortisol levels

would circumstantially link GR activation with immunosuppression in periparturient dairy cows.

III. MATERIALS AND METHODS

A. Cows and Blood Collections

Eight multiparous and 5 primiparous Holstein cows were used as periparturient test animals, and 5 multiparous and 5 primiparous midgestation Holsteins as non-periparturient control animals. All animals were housed at the Michigan State University (MSU) Dairy Teaching and Research facility and managed by the facility's herdsmen. Use of animals for this study was approved by MSU's All University Committee for Animal Use and Care.

Blood samples for flow cytometric analysis of lymphocyte and monocyte GR expression and leukocyte counting were collected by tail venipuncture into Vacutainer brand blood collection tubes containing 1.0 ml of acid citrate dextrose anticoagulant [p, q] using 20 *g* 2.5-cm multi-sample needles [o]. Samples for serum harvesting (cortisol assay) were collected into Vacutainer tubes that contained no anticoagulant [r].

The original schedule for blood collections from periparturient test cows included 11 samples relative to expected calving dates, on days –28, -21, -14, -7, hours 0, 12, 24, 36, 48, and days 7, and 14. Pre- and postpartum daily samples were collected at 0800 h, while the intense sampling around calving occurred at precise hours relative to the 0 h (calving) sample. While 5 animals calved precisely on schedule, the remaining 8 cows calved earlier than expected. In these cases, prepartum days relative to calving were assigned retrospectively and labeled according to closest day (-21 or –14 d) in the original sampling schedule. In the end, all 13 test cows had data from –14 d on, while 7 test cows had data for –21 d and 5 cows for –28 d. Therefore, peripartum data more accurately reflected week relative to calving for these cows. Calving dates were also staggered over a 2-month period (October to December, 1997), with multiparous cows calving first followed by primiparous cows. To insure that adequate control samples were obtained to

account for parity and season effects and for assay errors over time, blood samples were collected from at least one parity-matched control animal every time a test animal was sampled. Therefore, there were more total observations for control cows (n = 141) than for test cows (n = 129) in any data set of this study.

B. Leukocyte Counting and Standardization of Cell Numbers for GR Staining

Total leukocytes were counted to standardize the number of cells added per well of 96-well U-bottom microtiter plates [t, u] for fluorescent GR staining, as well as for future use in determining mononuclear leukocyte counts. Red blood cells from 5 ml of whole blood were lysed for 1.5 min using 10 ml cold hypotonic lysing solution [cc] (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, pH 7.3). This was followed by addition of 20 ml cold hypertonic restoring solution [dd] (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, 0.43 M NaCl, pH 7.3). Remaining leukocytes were centrifuged [y, used for all centifugation] at 1000 x *g* for 5 min at 22^0 C and supernatants aspirated. The cell pellet was washed twice in 2 ml cold PBS [ee] and centrifuged at 800 x *g* for 5 min at 22^0 C. Leukocytes were counted on a hemacytometer [z] and adjusted to 5 x 10^6 cells per well, and subjected to fluorescence staining (below).

C. Fluorescent Staining of Mononuclear Leukocyte GR

Prepared leukocytes (above) were differentiated by plasma membrane expression of CD45 and GR was monitored in CD45-differentiated mononuclear leukocytes following fluorescent labeling. CD45 is a signal transduction molecule that is required for lymphocyte activation (Trowbridge and Thomas, 1994). It is a transmembrane protein that has different expression levels in various leukocyte populations (highest on lymphocytes, intermediate on monocytes, and lowest on neutrophils) and, therefore, is used as a leukocyte differential in fluorescence activated flow cytometry (Sutherland et al., 1994;

Trowbridge and Thomas, 1994). In the present study, leukocyte CD45 was immunolabeled with a primary antibody [i] and visualized using a phycoerythrin (PE)-conjugated anti-IgG_{2a} secondary antibody [e]. Therefore, lymphocytes were differentiated from monocytes in this study by the degree of PE fluorescence intensity. Labeled dexamethasone is commonly used to identify GR in target cells (Rupprecht, et al. 1993; Gagne, et al. 1986) because of its specificity and high affinity for GR. Fluorescein isothiocyanate (FITC)-conjugated dexamethasone [c] was used at 1×10^{-5} M in the current study because this concentration saturated the GR of 5×10^{6} cells per well in preliminary studies (data not shown). Specificity of GR labeling was demonstrated when 1000 fold molar excesses of the glucocorticoid antagonists, progesterone and RU486 (both from Sigma Chemical Co., St. Louis, MO) successfully competed with FITC-dexamethasone in this assay (not shown). The dual-color CD45/GR staining protocol was described in detail by Preisler et al., (1999).

D. Flow Cytometric Acquisiton of Mononuclear Leukocyte GR Expression Data

A FACSCalibur fluorescence activated flow cytometer [w] and the acquisition package of CellQuest software [v] were utilized to acquire lymphocyte and monocyte GR expression data. First, unstained cells were used to check and adjust the forward scatter (FSC = size) and side scatter (SSC = granularity) characteristics of leukocytes in FSC-SSC density plots (Figure 4.1a). Next, single-color stained cells were used to set FL-1 (FITC) and FL-2 (PE) detectors and compensation for the two-color analyses. FL-1, FL-2, and compensation settings were not altered during the actual trial. FITC and PE negative control cells were used to set background fluorescence. Finally, data from the dual-labeled cells were collected as the lymphocyte and monocyte GR expression data sets used for subsequent hypothesis testing. Data from 5000 leukocytes per sample per cow were acquired.

E. Flow Cytometric Analysis of Mononuclear Leukocyte GR Expression Data

The analysis package of CellQuest software [v] was used to determine lymphocyte and monocyte GR expression, which was recorded as FITC mean fluorescence intensity (MFI) of CD45-differentiated leukocytes. Mononuclear leukocytes were differentiated into lymphocytes and monocytes using both their granularity and CD45 staining characteristics (Figure 4.1b). Glucocorticoid receptor expression was then assessed from FITC fluorescence histograms of the differentiated leukocytes (Figure 4.1c). Periparturient changes in mononuclear GR expression were observed as shifts in MFI along the X-axis FITC histograms over time, as is demonstrated in Figure 4.2 for changes in lymphocyte GR expression around calving for one representative test cow. Therefore, lymphocyte and monocyte FITC MFI values were the data sets used to assess effects of parturition on GR expression (see below).

F. Leukocyte Counts

Lymphocyte and monocyte counts (number of cells per ml of blood) were determined by multiplying total leukocyte counts (number of leukocytes per ml of PBS; determined by hemacytometer) by the percentage (%) of CD45-differentiated lymphocytes and monocytes in a sample (determined by flow cytometry).

G. Blood Cortisol Concentrations

Cortisol concentrations were determined by radioimmunoassay (RIA) using a commercial Coat-A-Count Cortisol Determination Kit [b]. Sera were harvested from each blood sample following blood clotting and centrifugation of tubes [r] at 1000 x *g*, and were stored at –20^{o}C until use in the RIA. All sera were assayed in duplicate on 1 d and data were recorded as cpm using a Gamma Trac 1290 [x]. The cpm were then converted to

micrograms of cortisol per deciliter of serum (ug/dL) based on a standard curve of cpm by concentration. The RIA intraassay coefficient of variation was 0.65%.

H. Statistical Analyses

The Statistical Analysis Software (SAS) PROC MIXED was used to analyze data sets by mixed model analysis (SAS, 1996). The mixed model applied to all data sets included a random cow effect and fixed effects of parity, time (relative to calving), treatment group (control vs. test), calendar week of sampling (accounted for seasonal and assay variation over time), and a fixed time-by-parity interaction, as described in detail by Preisler et al. (1999). Because of the design of the study, other two-way interactions were confounded and were not included in the model. Upon initial examination of the distribution of GR expression data sets, log transformations were applied to MFI values. Cortisol and leukocyte count data sets were analyzed without transformation. Time and time-by-parity least squares means (LSMs) and standard errors of the means are reported for lymphocyte and monocyte GR expression (log MFI), cortisol (micrograms per deciliter), and leukocyte, lymphocyte, and monocyte counts (number of cells per milliliter). The correlation procedure of SAS (SAS, 1990) was used to determine associations between lymphocyte and monocyte GR expression and blood cortisol concentrations, leukocyte, lymphocyte, and monocyte counts. All available data from control and test cows are reported. Statistical significance was considered at $P \leq 0.05$.

IV. RESULTS

A. GR Expression in Bovine Mononuclear Leukocytes

Fluorescence staining of lymphocytes and monocytes indicated that, on average, monocytes had approximately twice the GR expression of lymphocytes (Figure 4.1c). The MFI values represented the geometric mean of the FITC intensity peaks for each leukocyte type (as indicated by the mean channel fluorescence on the X-axis in Figure 4.1c) contained

in 5000 total leukocytes counted from each blood sample. Studies in humans also demonstrated twice as many GR molecules in monocytes as in lymphocytes (Distelhorst and Benutto, 1981; Lipman and Barr, 1977; Schlecte et al., 1982; Shipman et al., 1983).

B. GR Expression in Lymphocytes of Periparturient Cows

Fluorescence histograms from multiple periparturient blood samples for one test animal (Figure 4.2) demonstrate shifts to the left of the 0 h (calving) and the 24 h histograms relative to the precalving (-21 d) histogram, as well as incomplete recovery of the GR expression peak by 7 d postcalving. Parturition induced dramatic and chronic GR down-regulation in circulating lymphocytes from this animal. Similarly, MFI of all lymphocytes from periparturient test animals at calving were lower than precalving MFI. Analysis of variance of lymphocyte GR expression revealed significant effects of time, calendar week, and the parity-by-time interaction (Table 4.1). As demonstrated by the time LSMs in Figure 4.3a, parturition caused a significant reduction ($P < 0.0001$) in FITC-dexamethasone staining in lymphocytes which started during the week prior to calving. In Figure 4.3, time relative to parturition on the X-axis is reported in days to provide accuracy in data spacing over time, therefore, 12 h equals 0.5 d. When converted back to the original unit of measurement (MFI), lymphocytes from test animals at calving had a mean 42% reduction ($P < 0.0001$) in GR expression relative to the mean GR expression in lymphocytes from control animals. The reduction in lymphocyte GR expression persisted to 24 h ($P < 0.0001$) and remained significantly lower ($P < 0.05$) than control GR values throughout the remaining 14 d postcalving period (Figure 4.3a). When compared with precalving values (mean of –28 d, -21 d, -14 d, and –7 d) of the test animals themselves, GR expression was reduced by 67% at calving.

C. GR Expression in Monocytes of Periparturient Cows

Analysis of variance of monocyte GR expression revealed significant effects of time, calendar week, and the parity-by-time interaction (Table 4.1). Time LSMs in Figure 4.3b demonstrated that parturition caused a significant reduction ($P < 0.0001$) in monocyte GR expression. By calving, monocyte GR down-regulation (MFI) reached a nadir that was 47% lower ($P < 0.0001$) than GR expression in monocytes of control animals and 54% lower ($P < 0.0001$) than the precalving mean (–28 d, -21 d, -14 d, and –7 d) GR expression in the test cows. Significant ($P < 0.0001$) down-regulation of monocyte GR expression persisted to 24 h postcalving and remained lower ($P < 0.05$) than control GR values throughout the remaining 14 d of the study.

D. Blood Cortisol Concentrations

The main effect of time relative to parturition was significant ($P < 0.0001$; Table 4.1) for blood cortisol concentrations. Time LSMs for cortisol were increased nearly seven fold at calving (0 h) compared to control and precalving means (Figure 4.3c), but the elevation was short-lived and returned to basal concentrations by 24 h postcalving. Cortisol concentration was negatively correlated with lymphocyte GR expression ($r = -0.213$; $P < 0.05$) and monocyte GR expression ($r = -0.253$; $P < 0.05$) overall.

E. Parity-By-Time Interaction Effects on Mononuclear Leukocyte GR Expression

The parity-by-time interaction effect was significant (Table 4.1) for both the lymphocyte GR ($P < 0.004$; Figure 4.4a) and monocyte GR ($P < 0.003$; Figure 4.4b) expression data sets. Parity-by-time LSMs in Figure 4.4 demonstrated that mononuclear leukocytes from primiparous cows had earlier, more acute reductions in GR expression, followed by more rapid increases in expression to 36 h postcalving than leukocytes from

multiparous cows. Glucocorticoid receptor down-regulation was more gradual and persistent in the multiparous cows.

F. Lymphocyte and Monocyte Counts

Significant time relative to parturition effects were observed for monocyte counts ($P < 0.01$) but not for lymphocyte counts ($P > 0.53$) (Table 4.1). Time LSMs in Figure 4.5 demonstrated that parturition increased the total number of circulating leukocytes, which was associated with neutrophilia at that time (Preisler et al., 1999). Parturition did not alter the number of circulating lymphocytes and only modest elevations in monocyte counts were observed 48 h postcalving ($P < 0.03$). In this study, the main effect of calendar week was significant for lymphocyte and monocyte counts ($P < 0.001$, $P < 0.006$; Table 4.1). There was also a significant parity effect ($P < 0.01$) on monocyte counts that resulted in multiparous test cows having higher overall circulating monocyte counts (5.2×10^6 cells/ml) on average that primiparous test cows (3×10^6 cells/ml). No correlations existed ($P > 0.10$) between GR expression or cortisol concentration and mononuclear leukocyte counts.

V. DISCUSSION

It is difficult to manage dairy cows during the peripartum period because of parturition-associated increases in metabolic disorders and infectious diseases, including mastitis. The natural stress of calving normally occurs several times in a particular cow's productive lifetime, and with each parity the incidence and severity of disorders increases (Curtis et al., 1985; Grohn et al., 1995). Also, parturition is associated with dysfunction of most leukocytes including altered expression of key proteins involved in trafficking, migration, antigen presentation, and activation (Burton and Kehrli, 1996; Burton et al., 1995; Kehrli et al., 1989a; Kehrli et al., 1989b; Kehrli et al., 1998; Lee and Kehrli, 1998; Nonnecke et al., 1997; Roth and Kaeberle, 1982). The exact mechanisms by which

leukocyte functions are altered around parturition are unknown. The findings of this study provide preliminary data that begin to support a potential role for GR regulation in periparturient immunosuppression related to leukocyte dysfunction and disease susceptibility. The observed reductions in lymphocyte and monocyte GR expression at calving in this study, and negative correlations between mononuclear cell GR expression and blood cortisol concentration, suggest that cortisol-activated GR might be involved in altered gene transcription in these cells around parturition.

Glucocorticoid receptor down-regulation observed for bovine mononuclear leukocytes in this study started in the week prior to calving. As demonstrated in Figures 4.4a and 4.4b, most of this decline in GR was attributed to mononuclear leukocytes of the primiparous cows and occurred slightly before increases in blood cortisol were detectable (Figure 4.3). Therefore, it is possible that other reproductive or nutrient partitioning hormones (or both) can precipitate GR down regulation in bovine leukocytes of primiparous cows. Although this possibility requires substantiation, reductions in mean FITC MFI on –7 d relative to –28 d, -21 d, and –14 d for all cows were modest and were not statistically different from the mean values for control cows. Therefore, virtually all of the detected "parturition effects" (i.e., parturient vs. control cows) on mononuclear leukocyte GR expression began at the 0 h (calving) sample in this study, most likely in association with the seven fold increase in blood cortisol.

Literature on GR regulation in species other than cattle offers several explanations for the parturition-induced GR down-regulation observed in the present study. Receptor degradation following GC-activation of GR partly explains homologous down-regulation of the receptor protein in many human cells, including lymphocytes (Burnstein and Cidlowski, 1992; Oakley and Cidlowski, 1993). If degradation of the GR protein occurred in response to elevated cortisol in the current study, fewer GR molecules would have been available to bind with FITC-dexamethasone and could have resulted in the reduced FITC MFI values. In fact, it appears that homologous GR down-regulation occurs at many

levels in all GC-sensitive tissues studied so far in humans and rodents (Dong et al., 1988; Schlecte et al., 1982; Shipman et al., 1983). Therefore, it is not unreasonable to speculate that the GR down-regulation observed bovine mononuclear cells in this study was also due to homologous down-regulation in response to cortisol secretion around calving. In addition to GR protein degradation, literature on humans cites clear evidence that the rate of GR gene transcription is reduced by GC-activated GR, leading to 30 to 80% reductions in GR mRNA abundance that readily explain reductions in receptor protein abundance (Burnstein and Cidlowski, 1992; Okret et al., 1986; Petrichenko et al., 1989; Rosewicz et al., 1988). The possibility that this also occurs in mononuclear leukocytes of periparturient cows is currently being explored in our laboratory as a means of explaining the observed reductions in FITC-dexamethasone binding, presumably indicating homologous GR down-regulation. All of these possibilities have implications for GR being involved in both its own autoregulation and the transcriptional regulation of other molecules in bovine lymphocytes and monocytes that are critical for effective immune responses. These possibilities should be explored in periparturient dairy cows as a means of explaining disease susceptibility around calving.

Evidence has been documented in studies using periparturient dairy cows and cattle treated with dexamethasone that leukocyte functions and phenotypes are dramatically altered when blood GC concentrations are high (Burton et al., 1995; Kehrli et al., 1989a; Kehrli et al., 1989b; Kehrli et al., 1998). For example, parturition impairs neutrophil phagocytic functions (Cai et al., 1994; Nagahata et al., 1988; Roth and Kaeberle, 1982) and expression of the CD62L adhesion molecule (Lee and Kehrli, 1998), both which facilitate important protective roles of neutrophils when the mammary gland becomes infected with mastitis causing bacteria. It is not surprising then, that the periparturient period is one of high mastitis susceptibility (Oliver and Sordillo, 1988). Decreased neutrophil CD62L expression and increased mastitis susceptibility are also observed when cattle are injected with dexamethasone (Burton and Kehrli, 1995; Burton et al., 1995).

Indeed, mastitis susceptibility is a general phenomenon of dexamethasone-treated cows (Lohuis, et al. 1988; Wesley, et al. 1989). Dexamethasone also has profound effects on mononuclear cells in cattle, including inhibition of IFN-γ and IgM secretion (Nonnecke et al., 1997) and decreased expression of MHC I and MHC II molecules (Burton and Kehrli, 1996). Because dexamethasone binds with high affinity to GR, these alterations in key leukocyte functions and phenotypes imply that GR is involved in dysfunctions of the bovine immune system during periods of GC challenge. Our mononuclear leukocyte GR expression and blood cortisol data suggest that parturition is one of these periods in dairy cows.

Parity differences in this study showed that multiparous animals had gradual but prolonged lymphocyte and monocyte GR down-regulation when compared to primiparous animals. Primiparous cows remained devoid of metabolic disorders or mastitis, whereas over half of the multiparous animals developed some combination of these disorders. This was not surprising because multiparous cows have higher incidences of health problems associated with parturition than primiparous cows (Curtis et al., 1985; Grohn et al., 1995). Because of low animal numbers in this study, statistical analyses of health effects on leukocyte GR expression were not attempted. However, exaggerated persistency of GR down-regulation in multiparous test cows could indicate that mononuclear leukocytes of these animals were more sensitive to parturition-related increases in blood cortisol than cells from primiparous animals. If GR regulation in peripheral tissues, including leukocytes, is related to stress adaptation and leukocyte dysfunction this could facilitate disease susceptibility. On the other hand, the stress of overcoming parturition in conjunction with the presence of metabolic disorders could have resulted in prolonged reduction of lymphocyte and monocyte GR expression in multiparous cows. Another related possibility is that higher milk production demands of multiparous versus primiparous cows could have stressed homeostasis to a larger degree resulting in exaggerated GR down-regulation, even in the face of normal cortisol concentrations beyond 24 h into lactation. Certainly effects of

other reproductive and nutrient partitioning hormones, and of disease, on leukocyte GR expression, function, and phenotype warrants further investigation. In any case, the degree of lymphocyte and monocyte GR down-regulation following parturition may be a better indicator of stress than blood cortisol concentrations and offers a possible explanation for the higher incidence of metabolic disorders and mastitis susceptibility in multiparous versus primiparous periparturient dairy animals. These possibilities require substantiation through experiments designed specifically to assess the role of GR down-regulation in gene expression (phenotype) and functional capacity of leukocytes, as well as in peripheral tissue sensitivity to homeostatic and homeorhetic hormones in periparturient dairy cows.

VI. CONCLUSIONS

The present study demonstrated glucocorticoid receptor down-regulation in bovine peripheral blood lymphocytes and monocytes at parturition in association with elevated maternal blood cortisol concentrations. Observed glucocorticoid receptor down-regulation has potential implications regarding possible mechanisms regulating immunocompetence and disease susceptibility around parturition, especially in multiparous cows.

Chapter Four

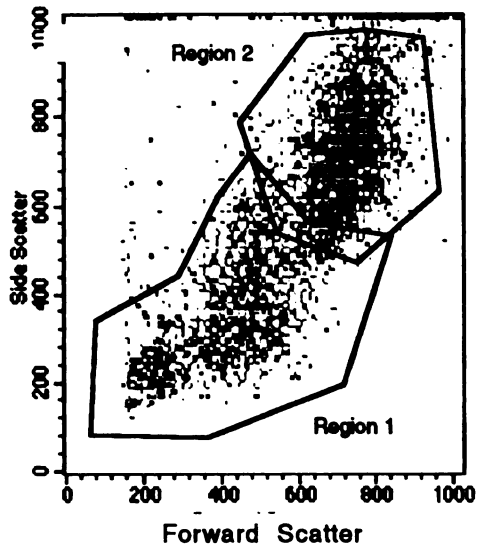
Tables and Figures

TABLE 4.1 – *P* -values for terms in the mixed model analysis of variance of mononuclear leukocyte glucocorticoid receptor (GR) expression, serum cortisol concentration, and total counts of lymphocytes and monocytes.

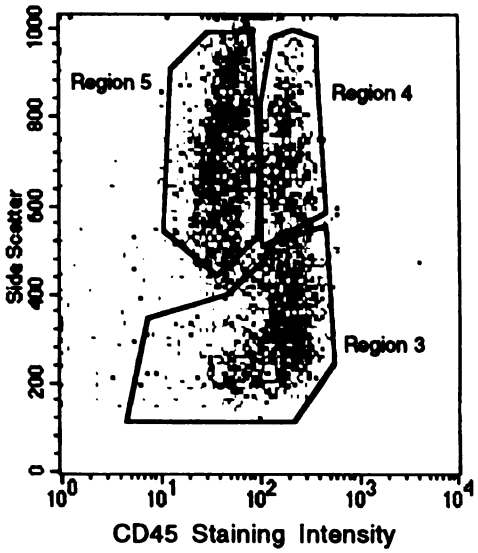
	Mononuclear Leukocyte GR Expression		Cortisol Conc.	Mononuclear Leukocyte Counts	
Model Effect	Monocytes (Log of MFI)	Lymphocytes (Log of MFI)	Cortisol (ug/dL)	Lymphocytes (# cells/ml)	Monocytes (# cells/ml)
Time	0.0001	0.0001	0.0001	0.5309	0.0099
Parity	0.3788	0.2894	0.1571	0.7235	0.0091
Calendar Wk.	0.0046	0.0001	0.7174	0.0001	0.0059
Parity x Time	0.0023	0.0036	0.1435	0.3075	0.6196

Figure 4.1- Leukocyte differentials were performed by flow cytomtery using a combination of cellular granularity and size (a), and cellular granularity and CD45 staining intensity (b). Leukocytes that gated within Regions 1 + 3 were identified as lymphocytes, within Regions 2 + 4 as monocytes, and within Regions 2 + 5 as neutrophils. Mean FITC staining intensity of doubly gated lymphocytes and monocytes were used to determine GR expression (c), which was recorded as the geometric mean channel fluorescence (or mean fluorescenec intensity, MFI) of each histogram peak. Representative FITC histograms for one control animal show that circulating lymphocytes (MFI = 35.60) express approximately half as much GR as monocytes (MFI = 73.09).

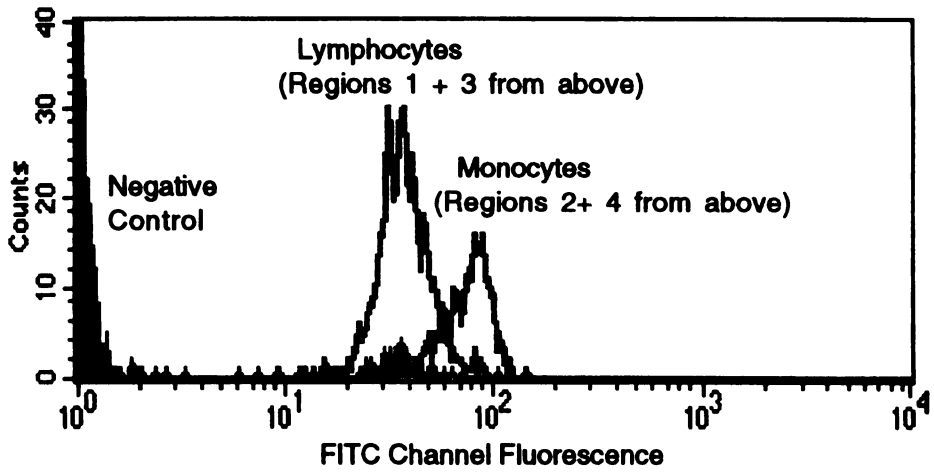
(a) **Density Dot Plot of Leukocyte Granularity vs. Size**



(b) **Density Dot Plot of Leukocyte Granularity vs. CD45-Staining**



(c) **Fluorescence Histogram of Leukocyte Count vs. FITC-Staining Intensity**



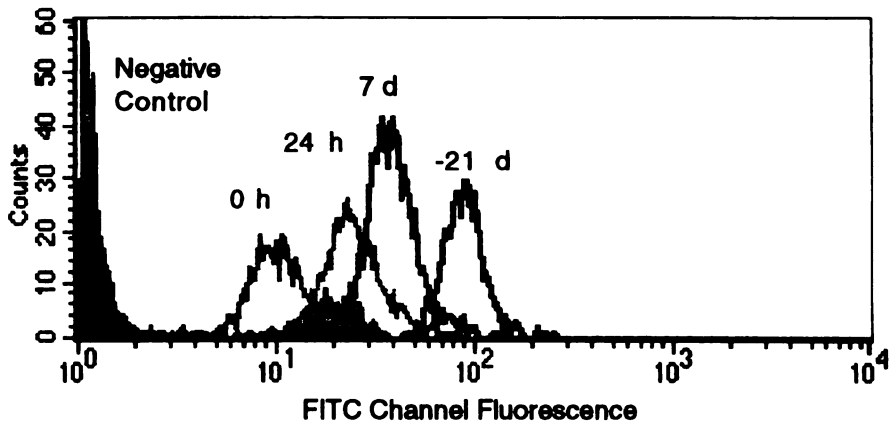
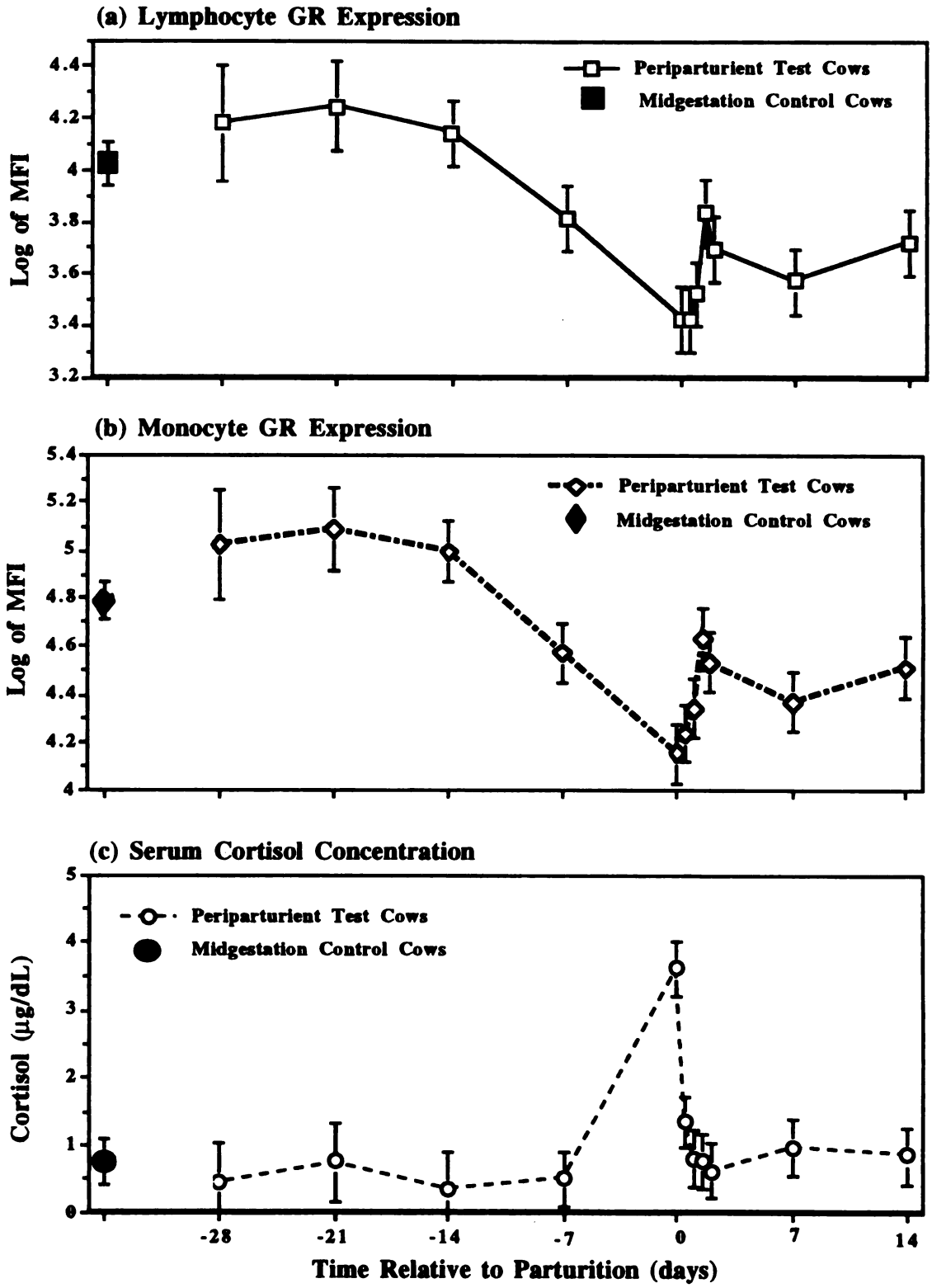


Figure 4.2- Parturition-induced GR down-regulation was determined by shifts in the geometric mean channel fluorescence (MFI) of FITC-dexamethasone stained mononuclear leukocytes. Representative FITC histograms (open peaks) for lymphocytes of one test animal are shown for the following sample times: 21 d pre-calving (-21 d), calving (0 h), 24 h post-calving (24 h), and 7 d post-calving (7 d). Background fluorescence (negative control) is shown in the solid histogram.

Figure 4.3- Time least squares means (± SEM) of lymphocyte GR expression (a), monocyte GR expression (b), and serum cortisol concentrations (c) in periparturient test cows (open symbols) and midgestation control cows (solid symbols). Parturition occurred at time 0 h.


(a) Lymphocyte GR Expression
Periparturient Test Cows
Midgestation Control Cows
Log of MFI
4.4
4.2
4
3.8
3.6
3.4
3.2
(b) Monocyte GR Expression
Periparturient Test Cows
Midgestation Control Cows
Log of MFI
5.4
5.2
5
4.8
4.6
4.4
4.2
4
(c) Serum Cortisol Concentration
Periparturient Test Cows
Midgestation Control Cows
Cortisol (µg/dL)
5
4
3
2
1
0
-28
-21
-14
-7
0
7
14
Time Relative to Parturition (days)

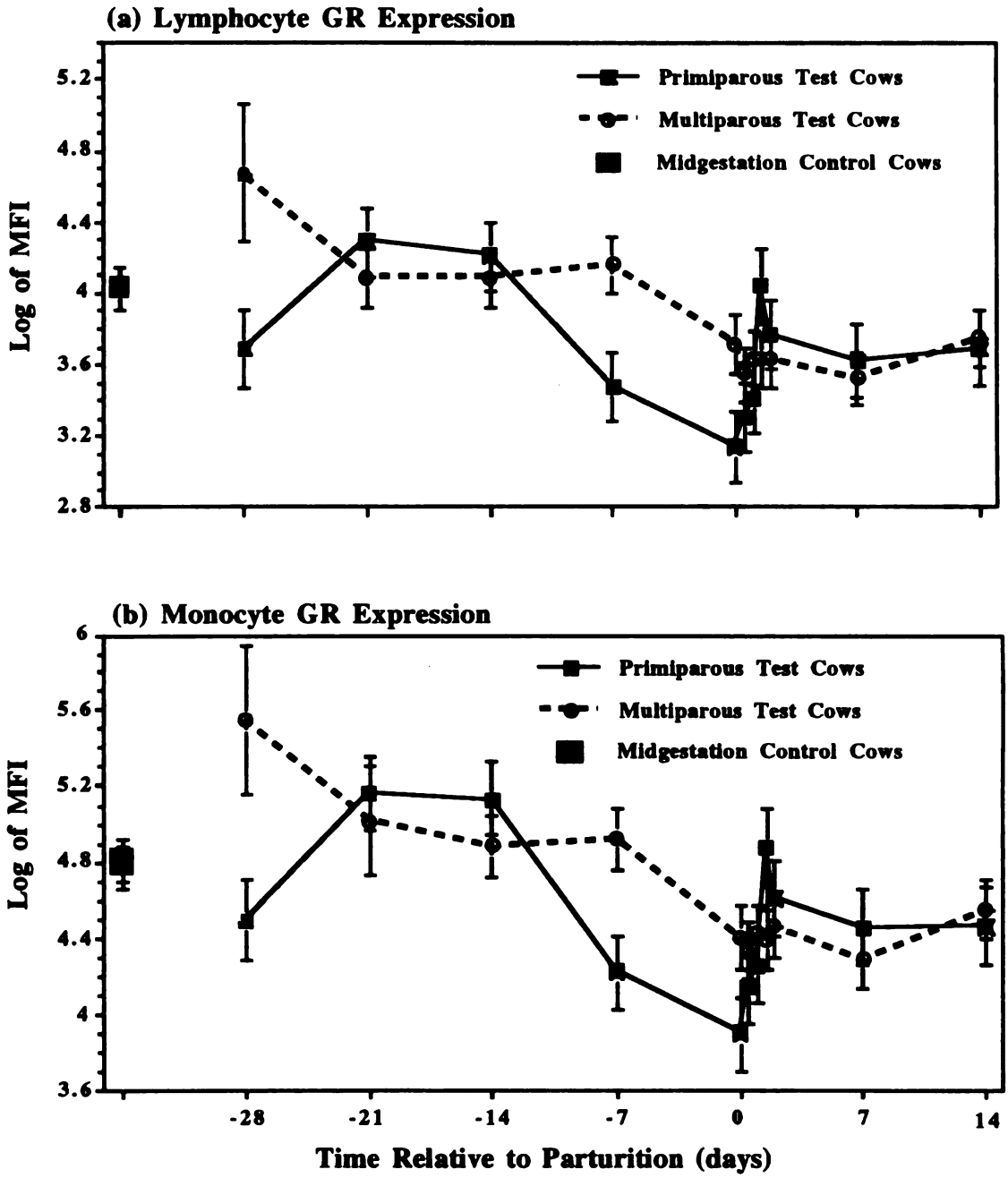


Figure 4.4- Parity-by-time least squares means (± SEM) of lymphocyte (a) and monocyte (b) GR expression in primiparous test cows (x'ed squares), multiparous test cows (x'ed circles), and midgestation control cows (solid symbols). Parturition occurred at time 0 h.

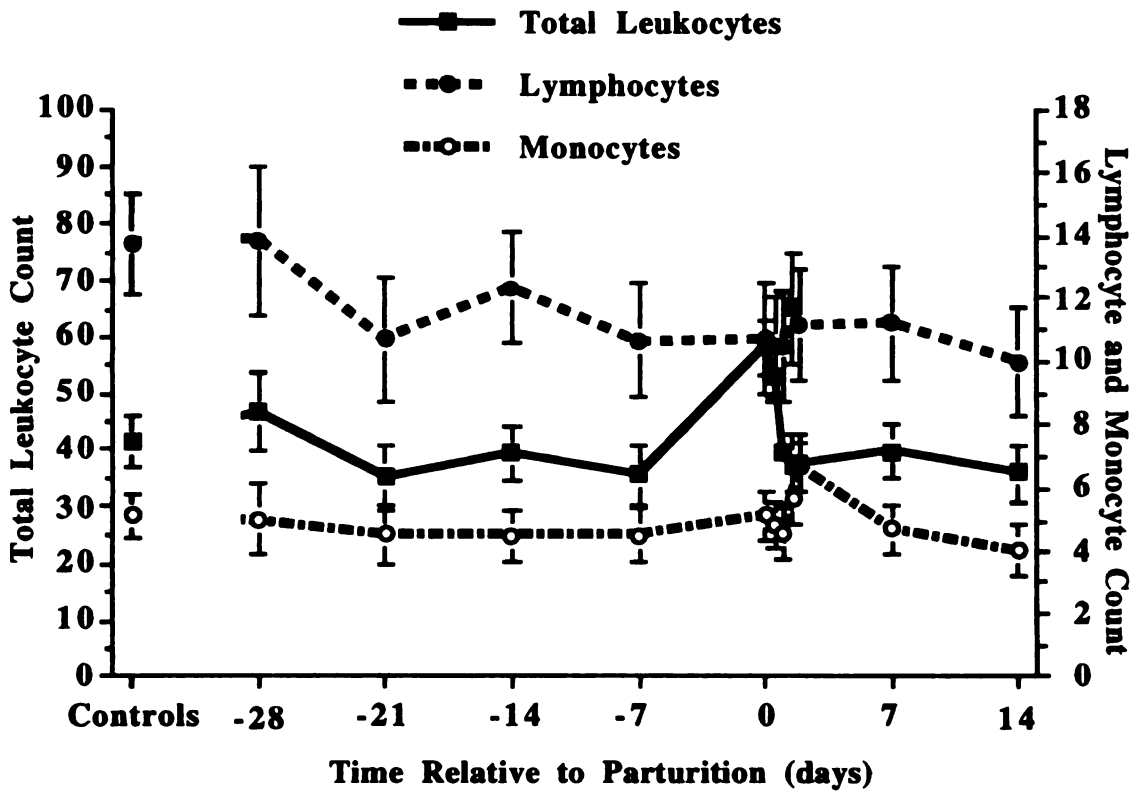


Figure 4.5- Time least squares means (± SEM) for total leukocyte (solid squares), lymphocyte (solid circles),and monocyte counts (open circles) in periparturient test cows (lines) and midgestation control cows (single symbols). Parturition occurred at time 0 h.

CHAPTER FIVE

Discussion

It has been speculated that the higher incidences of production-related diseases in dairy cows may be associated with the increased metabolic demands for higher milk yield per cow (Grohn et al., 1995). Of particular interest has been the increase in the incidence of mastitis among high-yielding animals, especially multiparous cows. These cows are not only more susceptible to mastitis, but also to other infectious and metabolic diseases (Curtis et al., 1985; Grohn et al., 1995). The risk of mastitis is much higher during the periparturient period, a time when the animal encounters profound physical, metabolic, nutritional, and hormonal changes that may be stressful and alter immunocompetence. One potent immune stressor in periparturient cows is cortisol, a GC that impairs most aspects of innate immunity and has both a suppressing and enhancing effects on acquired immunity (refer to Table 1.2, Chapter One). Indeed, the several-fold increases in blood concentrations of cortisol in periparturient cows led to our hypothesis that bovine leukocytes possess the receptor for cortisol (GR), and that cortisol-activated GR may be involved in periparturient immune suppression (Goff et al., 1989; Guidry et al, 1976). To begin to test these hypotheses, we developed a flow cytometric assay that simultaneously differentiated leukocytes and monitored GR expression (Chapter Two). We used this assay to monitor bovine leukocyte GR expression during the periparturient period (Chapters Three and Four).

Results from the periparturient study reported in this thesis demonstrated down-regulated GR expression in neutrophils (Chapter Three), lymphocytes (Chapter Four),

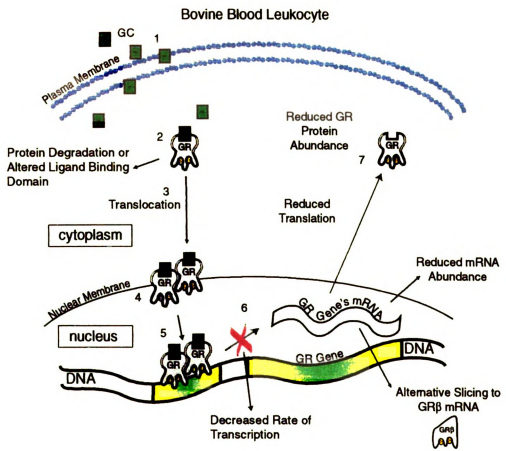


Figure 5.1 – Proposed mechanisms for homologous GR down-regulation in bovine blood leukocytes.

and monocytes (Chapter Four) at parturition in association with a mean 7-fold increase in blood cortisol concentration. There are several possible explanations for the decrease in GR expression. Figure 5.1 depicts the possible regulatory pathway of GR expression that includes simple GR translocation from the cytoplasm into the nucleus, protein degradation of the GC-activated receptor, and transcriptional, translational, and (or) post-translational regulation of GR expression. A change at any one of these steps could have direct effects on the function, phenotype, and expression of GR, which ultimately may change sensitivity of the leukocytes to GC. In addition, while GR may regulate itself, the cortisol-activated receptors are also predicted to regulate transcription of other genes that they normally affect (such as CD62L – Lee and Kehrli, 1998; and several cytokines - Almawi, et al., 1996). Therefore, GR down-regulation is expected to correlate with altered phenotypes and functions of GC-sensitive cells, such as seems to occur in bovine blood leukocytes. For example, data from this study suggests that when GR is down-regulated by high cortisol concentrations at calving, neutrophil trafficking is impaired leading to neutrophilia and leukocytosis. As depicted in Figure 5.1, the following is a summary of possible explanations for these observations of GR down-regulation.

To start, GC diffuses across the plasma membrane of leukocytes (Figure 5.1, step 1), and binds with cytosolic GR (step 2). Elevated GC concentrations have been shown to enhance GR protein degradation (reviewed in Oakley and Cidlowski, 1993), and this would mean that our assay would detect decreased FITC-DEX binding (ie. lower FITC fluorescence intensity), as was observed. Another possibility is that increased GC-GR binding could alter ligand-binding at the transcriptional (rate of GR gene transcription and (or) alternative splicing to GRβ) or post-transcriptional (GR mRNA stability) levels

in such a way as to inhibit further binding of GC (Dong et al., 1988). This too, would be detected as a decrease in GR (FITC-DEX labeling) by our assay. To determine if either possibility is valid, the development of bovine GR-specific monoclonal antibodies and RNA/DNA probes would be valuable. For example, a detection antibody for simple identification of GR proteins would substantiate the current results by both Western blot and flow cytometry. Antibodies that specifically recognize the alpha and beta isoforms of GR could also be valuable for determining if GC-GR binding actually causes alternative splicing of the GR gene to yield the beta form of GR (Oakley and Cidlowski, 1996) (Figure 5.1). GRβ lacks a ligand binding domain, which would also lead to a detectable decrease in GR (FITC-DEX labeling) by our assay. Another useful tool would be an antibody to block the ligand-binding domain to use in competition with agonists, like FITC-DEX, to determine binding specificity, measurable by a decrease in FITC-DEX binding to GR. While these scenarios of protein degradation/altered function partly explain GR down-regulation, the most impact of GC-activated GRs seems to be at the transcriptional level, at least in human and rodent studies (Burnstein and Cidlowski, 1992; Cidlowsi and Cidlowski, 1981; Dong et al., 1988; Guido et al., 1996; Kalinyak et al., 1997; Oakley and Cidlowski, 1993; Okret et al., 1996; Rosewicz et al., 1988)

After GC is bound to GR, the receptor dimerizes (step 3) and translocates to the nucleus (step 4) to bind DNA and regulate target gene transcription (step 5). Literature on GR regulation in many species offers explicit explanations for decreased GR expression in cells in the presence of glucocorticoids. There is substantial evidence that GR regulates its own gene expression at the transcriptional as well as protein level (Dong et al., 1988; Schlecte et al., 1982; Shipman et al., 1983). This homologous down-

regulation occurs in response to elevated GCs and has been observed at the protein level (Schlecte et al., 1982; Shipman at al., 1983), by detection with monoclonal antibodies (Dong et al., 1988), and at the mRNA level using molecular probes that detect mRNA abundance, rate of GR gene transcription, and isoform switching to GRβ (Burnstein and Cidlowski, 1992; Oakley and Cidlowski, 1996; Okret et al., 1986, Rosewicz et al., 1988). Therefore, it is not unreasonable to speculate that elevated blood cortisol concentrations at parturition also cause homologous GR down-regulation in bovine blood leukocytes. Decreased GR gene transcription (Figure 5.1, red "X") would lead to a decrease in GR mRNA abundance (Figure 5.1, step 6) that would correlate with decreased GR receptor numbers (step 7). According to the human literature, this could be due to both reduced rate of GR gene transcription and reduced stability of GR mRNAs. If either event were occurring in blood leukocytes of the periparturient animals in our study, there would be less GR protein in the cells' cytoplasm and less FITC-DEX binding. This would certainly explain the observed decrease in leukocyte GR expression documented in Chapters Three and Four. Therefore, a likely next step in this research would be to determine if there is a reduction in GR mRNA abundance (stability and (or) rate of transcription) (Figure 5.1, step 6) in leukocytes of periparturient cows. However, bovine specific RNA probes have limited this work until the sequence of the GR cDNA has been established. This work is being done in our laboratory (Weber and Burton, unpublished), and two bovine GR probes are now ready for use in Northern blot, slot blot, and nuclear run-on assays to assess transcriptional repression of GR by GC-activated GR. The exact mechanisms of GR regulation need to be resolved in cattle due to the implications of GR activation on leukocyte function, immunocompetence, and disease susceptibility.

Our study also observed leukocytosis and neutrophilia at parturition, and these correlated with the rise in blood cortisol and decreased leukocyte GR expression. Therefore, these preliminary data implicate GR activation as being partly responsible for increased mastitis (disease) susceptibility in periparturient dairy cows (Curtis et al., 1985; Grohn et al., 1995). In particular, multiparous cows were more prone to metabolic disorders in this study. This could imply that with increased parity, leukocytes become cortisol resistant due to the stress of parturition that leads to immune cells and tissues that don't respond to cortisol as well, or as quickly in multiparous cows as in primiparous cows. Parturition is associated with multiple leukocyte dysfunctions, including altered expression of molecules involved in trafficking, migration, antigen presentation, and activation (Refer to Table 1.3). For example, in both the DEX challenge model (Burton et al., 1995) and the natural GC challenge model (parturition) (Lee and Kehrli, 1998), the neutrophil adhesion molecules CD62L and CD18 had decreased surface expression that resulted in neutrophilia and leukocytosis. Also, in both GC challenge models there is altered mononuclear leukocyte functions as reviewed in Tables 1.2 and 1.3 of Chapter One. If GC-activated GR has an additional role of repressing transcription of these genes, both the innate (neutrophils) and adaptive (lymphocytes and monocytes) immune responses would be impaired leading to a state of generalized immunosuppression and disease susceptibility around calving. To our knowledge, this study was the first to determine that parturition, and its associated increases in blood cortisol, results in decreased leukocyte GR expression that correlates with traits of altered leukocyte (neutrophil) trafficking. Mononuclear leukocyte trafficking was not affected during GR down-regulation but certainly, other indicator traits should be monitored in future studies

(eg. MHC expression). While the precise mechanism(s) for decreased GR expression have yet to be elucidated, these data set the stage for future experiments to study the molecular mechanisms of GR autoregulation and transcriptional regulation of other important genes used by leukocytes to defend against infectious disease pathogens. With this knowledge, leukocyte GR expression could become a useful phenotypic marker that could be used in breeding selection schemes to change leukocyte responsiveness to GC in periods of stress, or in the development of pharmaceuticals to block the negative effects of GR on GC responsive leukocytes.

CHAPTER SIX

Recommendations for Future Research

As mentioned in previous chapters, ligand-activated GR translocates to the nucleus where it binds promoters and other regulatory regions of GC-responsive genes and regulates their transcription. If the bovine GR gene is auto-regulated in leukocytes and decreases GR protein levels in response to elevated GC, as the results of the current study imply, then determining if GR mRNA abundance is reduced in leukocytes from parturient cows would support our observations of GR down-regulation. To test the hypothesis that GR mRNA expression is decreased in leukocytes of periparturient cows, bovine specific GR probes will need to be developed. Our lab has cloned and sequenced large fragments of bovine GR gene exon 2 (915 bp) and 9α (397 bp). These DNA fragments have been successfully labeled with the digoxygenin alkaline phosphatase system for chemiluminescent detection of GR mRNA by Northern blot analysis and slot blot analysis. Total RNA isolated from blood leukocytes has already been collected from 5 periparturient test heifers and 5 non-periparturient, mid-gestation control heifers following the same sampling schedule as the previous studies (Chapters Three and Four). To monitor GR mRNA abundance in leukocyte subsets, whole blood leukocytes were separated by Percoll density centrifugation into granulocytes and mononuclear cells. RNA was isolated by the Trizol method, and is now ready for Northern and slot blot analysis. The results of this study should be completed by December 1999 (Preisler, Weber, Madsen, and Burton). We anticipate that GR mRNA abundance will decrease for at least 48 h around calving in relative proportions to observed GR protein reductions in

corresponding leukocytes. If we are right, these results would significantly substantiate our notion that GR is involved in its own regulation in bovine leukocytes at the same time as contributing to generalized leukocyte dysfunctions and disease susceptibility in parturient cows. However, ultimate determination of reduced rate of transcription, by an assay such as nuclear run-on, would be the necessary experiment to be sure that GC-activated GRs actually alter GR expression transcriptionally. If so, new molecular drugs could be developed to "desensitize" leukocytes to GCs during known periods of stress by binding to GR mRNA or antagonizing the actions of cortisol.

Another good study would be to use our novel assay to monitor GR expression in a dexamethasone challenge model. Parturition is associated with changes of hormones other than GCs, such as the sex steroids (progesterone and estrogen) and nutrient partitioning hormones (insulin, growth hormone, and insulin-like growth factor I) that could have initiated or facilitated the decreased expression of leukocyte GR (observed at –7d). To eliminate this possibility, a study could be designed to test the hypothesis that administration of a potent and specific GC like DEX, alters GR protein and mRNA expression in bovine leukocytes. To determine the effects of GC administration may require DEX injections on three consecutive days to keep the level of this GC above basal concentrations (Burton and Kehrli, 1995). DEX concentrations would be expected to increase and correlate with a decrease in leukocyte GR expression. The same could be done using other hormones known to fluctuate in periparturient cows to determine their impact on leukocyte GR expression. If GR expression is not altered except by DEX treatment, it would show that GR is regulated only by GCs and that GCs are probably responsible for altering leukocyte functions via GR activation at calving. Another

interesting approach would be to take serum from periparturient cows, filtered over charcoal to remove GC, and test its activity on leukocyte GR down-regulation in vitro. This also may tell us what other hormones of parturient cows potentially affect bovine leukocyte GR.

To further understand how GR is related to immune dysfunctions, another study could be conducted to test the hypothesis that altered GR expression occurs in mastitis challenged cows and contributes to severity of the disease. It would be most informative if there were three groups of cattle included. These would include a control (no challenge) group, a mastitis challenged group to determine the effects of disease on GR expression, and a mastitis challenged group that received a GC injection or injections to monitor the combined effects of disease and GCs on GR expression. A variety of immunocompetence indicators could be monitored in addition to blood cortisol concentrations to correlate with any observed changes in GR expression. This type of study could also be applied to other natural stress situations, which could have significant implications for immunocompetence and increased disease susceptibility in these scenarios as well. For instance, production animals experience stress during dehorning, castration, puberty, estrus, shipping, handling, crowding, etc. All of these situations cause increases in blood cortisol concentrations, and leave animals more susceptible to infectious diseases, suggesting that GR could be involved.

An understanding of the dynamics between GR and its regulatory roles for its own gene and other important target genes involved in the immune response could open new avenues to the treatment and prevention of infectious diseases, in particular mastitis. It would be ideal that if GR is regulating important genes, its negative effects be

separated from its positive effects. For example, in a periparturient dairy cow, the increase in blood cortisol is necessary for the expulsion of the fetus and onset of lactation (positive effects) but, in contrast, there is a need to prevent GR from down-regulating the expression of the adhesion molecule CD62L (negative effect). This could be possible by the development of novel pharmaceuticals to treat or prevent the negative effects of GR on immune genes from occurring. The result would be decreased susceptibility and incidence of infectious diseases, in particular mastitis. This is the ultimate goal of mastitis researchers today.

APPENDIX ONE

Reagents, Supplies, Equipment, and Solutions

ID	Product	Company	City, State
	Reagents:		
a	B&D FACS Lysing Solution	Becton Dickinson Immunocytometry Systems	Mansfield, MA
b	Coat-A-Count Cortisol Determination. Kit	Diagnostics Products, Corp.	Los Angeles, CA
c	Dexamethasone Fluorescein	Molecular Probes	Eugene, OR
d	FITC Control IgG_1	Dako, Corporation	Carpenteria, CA
e	Goat α-Mouse IgG_{2a} -PE Clone M32204	Caltag Laboratories	Burlingame, CA
f	Hemalene II - Sheath Fluid	Bergen/Brunswich Medical Co.	Lake Zurich, IL
g	IgG_{2a}, Negative Control-RPE	Dako, Corporation	Carpenteria, CA
h	Mifepristone, RU486	Sigma Chemical, Co.	St. Louis, MO
i	Mouse α-Bovine CD45, IgG_{2a} Clone CACTB51A	VMRD	Pullman, WA
j	Permeablization Solution B	Caltag Laboratories	Burlingame, CA
k	Progesterone	Sigma Chemical, Co.	St. Louis, MO
l	Propidium Iodide	Sigma Chemical, Co.	St. Louis, MO
	Supplies:		
m	Falcon Tubes	Fisher Scientific	Pittsburgh, PA
n	Multichannel Pipettor Tips	Corning, Costar Corporation	Cambridge, MA
o	Vacutainer 20G1 Needles	Fisher Scientific	Pittsburgh, PA

Table A.1 continued

ID	Product	Company	City, State
p	Vacutainer Needle Holders	Fisher Scientific	Pittsburgh, PA
q	Vacutainer Tubes - Acid Citrate Dextrose	Fisher Scientific	Pittsburgh, PA
r	Vacutainer Tubes	Fisher Scientific	Pittsburgh, PA
s	50 ml Conical Tubes	Fisher Scientific	Pittsburgh, PA
t	96-Well Plates	Nalgene Nunc International	Milwaukee, WI
u	96-Well Sealing Tape	Nalgene Nunc International	Milwaukee, WI
	Equipment:		
v	CellQuest Software	Becton Dickinson Immunocytometry Systems	Mansfield, MA
w	FACSCalibur Flow Cytometer	Becton Dickinson Immunocytometry Systems	Mansfield, MA
x	Gamma Trac 1290	TM Analytic	Elk Grove, IL
y	GS-6R Centrifuge	Beckman Instruments, Inc.	Schamburg, IL
z	Hy-Lite Counting Chamber Hemacytometer	Hausser Scientific Company	Horsham, PA
aa	Leica DML	Leica Microscopy & Systems	Wetzlar, Germany
bb	Multichannel Pipettor	Corning, Costar Corporation	Cambridge, MA

Table A.1 continued

ID	Product	Company	City, State
	Solutions:		
cc	10.56 mM Hypotonic Lysis pH 7.3: in 1000 ml		
	Na_2HPO_4 1.499 g	J. T. Baker, Inc.	Phillipsburg, NJ
	NaH_2PO_4 0.368 g	J. T. Baker, Inc.	Phillipsburg, NJ
dd	10.56 mM Hypertonic Retore pH 7.3: in 1000 ml		
	Na_2HPO_4 1.5 g	J. T. Baker, Inc.	Phillipsburg, NJ
	NaH_2PO_4 0.4 g	J. T. Baker, Inc.	Phillipsburg, NJ
	NaCl 25.1 g	J. T. Baker, Inc.	Phillipsburg, NJ
ee	Phosphate Buffered Saline: in 1000 ml		
	Na_2HPO_4 8.00 g	J. T. Baker, Inc.	Phillipsburg, NJ
	NaCl 0.20 g	J. T. Baker, Inc.	Phillipsburg, NJ
	KCl 1.44 g	J. T. Baker, Inc.	Phillipsburg, NJ
	KH_2PO_4 0.24 g	J. T. Baker, Inc.	Phillipsburg, NJ
ff	10 mM Tris Incubation Solution: in 1000 ml		
	1M Tris, pH 7.6 10 ml	J. T. Baker, Inc.	Phillipsburg, NJ
	DMSO 10 ml	Sigma Chemical, Co.	St. Louis, MO
	BSA 1.0 g	Sigma Chemical, Co.	St. Louis, MO
gg	10 mM Tris Wash Solution: in 1000 ml		
	1M Tris, pH 7.6 10 ml	J. T. Baker, Inc.	Phillipsburg, NJ
	DMSO 10 ml	Sigma Chemical, Co.	St. Louis, MO
	BSA 1.0 g	Sigma Chemical, Co.	St. Louis, MO
	ETOH 50 ml	Quantum Chemical Company	Tuscola, IL

APPENDIX TWO

MICHIGAN STATE
UNIVERSITY

June 16, 1999

Dr. Gussie J. Tessier
Assistant Editor
American Journal of Veterinary Research
American Veterinary Medical Association
1931 N. Meacham Road, Suite 100
Schaumburg, IL 60173-4360

Dear Dr. Tessier:

I am writing to you in regards to the article "Glucocorticoid Receptor Down-Regulation in Neutrophils of Periparturient Cows" that has been accepted for publication in the *American Journal of Veterinary Research* (revised manuscript number 20679R). I am currently a Master's student at Michigan State University and am scheduled to defend my thesis on July 8, 1999. I request permission to include this manuscript as Chapter Three in my thesis.

Thank you for your prompt consideration of this matter!

Sincerely,

Mara T. Preisler

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Permission granted, provided that material is used only for the stated purpose.

Kurt Matushek 6/23/99

Kurt Matushek, DVM, MS
Associate Editor
Date

MICHIGAN STATE
UNIVERSITY

June 16, 1999

Dr. Stephen C. Nickerson
Editor
Physiology and Management Section of the *Journal of Dairy Science*
Hill Farm Research Station
Louisiana State University
Route 1, Box 10
Homer, LA 71040-9604

Dear Dr. Nickerson:

I am writing to you in regards to the article "Glucocorticoid Receptor Expression Profiles in Mononuclear Leukocytes of Periparturient Holstein Cows" that has been accepted for publication in the *Journal of Dairy Science* (revised manuscript number 9062-SCN). I am currently a Master's student at Michigan State University and am scheduled to defend my thesis on July 8, 1999. I request permission to include this manuscript as Chapter Four in my thesis.

Thank you for your prompt consideration of this matter!

Sincerely,

Mara T. Preisler

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John W Fuquay, Editor-in-Chief
"JOURNAL of DAIRY SCIENCE"
6/22/99

REFERENCES

Agarwal, M. K. 1996. The antiglucocorticoid action of mifepristone. Pharmacol. Ther. 70:183-213.

Almawi, W. Y., H. N. Beyhum, A. A. Rahme, M. J. Rieder. 1996. Regulation of cytokine and cytokine receptor expression by glucocorticoids. J. Leuk. Biol. 60:563-572.

Arya, S. K., F. Wong-Staal, R. C. Gallo. 1984. Dexamethasone-mediated inhibition of human T cell growth factor and _-interferon messenger RNA. J. Immunol. 133:273-276.

Ballard, P. L., J. D. Baxter, S. J. Higgins, G. G. Rousseau, G. M. Tomkins. 1974. General presence of glucocorticoid receptors in mammalian tissues. Endocrinol. 94:998-1002.

Bamberger, C. M., H. M. Schulte, G. P. Chrousos. 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Rev. 17:245-259.

Bloom, J. C., V. K. Ganjam, T. G. Gabuzda. 1980. Glucocorticoid receptors in peripheral blood lymphocytes from bovine leukemia virus-infected cows with persistent lymphocytosis. Cancer Res. 40:2240-2244.

Burnstein, K. L., J. A.Cidlowski. 1992. The down side of glucocorticoid receptor regulation. Mol. Cell. Endocrinol. 83:C1-C8.

Burton, J. L., M. E. Kehrli, Jr., S. Kapil, and R. L. Horst. 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J. Leuk. Bio. 57:317-325.

Burton, J. L., M. E. Kehrli, Jr. 1995. Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol- and dexamethasone-treated cows. Am. J. Vet. Res. 56:997-1006.

Burton, J. L., M. E. Kehrli, Jr. 1996. Effects of dexamethasone on bovine circulating T lymphocyte populations. J. Leuk. Biol. 59:90-99.

Burvenich, C., M. J. Paape, A. W. Hill, A. J. Guidry, R. H. Miller, R. Heyneman, W. D. J. Kremer, A. Brand. 1994. Role of neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. Vet. Quarterly. 16:45-50.

Cai, T-Q., P. G. Weston, L. A. Lund, B. Brodie, D. J. McKenna, W. C. Wagner. 1994. Association between neutrophil functions and periparturient disorders in cows. Am. J. Vet. Res. 55:934-943.

Carslon, G. P., J. J. Kaneko. 1976. Influence of prednisolone on intravascular granulocyte kinetics of calves under nonsteady state conditions. Am. J. Vet. Res. 37:149-151.

Carson-Jurica, M. A., W. T. Schrader, B. W. O'Malley. 1990. Steroid receptor family: Structure and functions. Endocrine Rev. 11:210-217.

Celada, A., S. McKercher, R. A. Maki. 1993. Repression of major histocompatability complex IA expression by glucocorticoids: the glucocorticoid receptor inhibits the DNA binding of the X box DNA binding domain. J. Exp. Med. 177:-691-698.

Chrousos, G. P. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. New England J. Med. 332:1351-1362.

Cidlowski, J. A., N. B. Cidlowski. 1981. Regulation of glucocorticoid receptors by glucocorticoids in cultured HeLa S3 cells. Endocrinol. 109:1975-1982.

Curtis, C.R., H. N. Erb, C. J. Sniffen, R. D. Smith, D. S. Kronfeld. 1985. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. J. Dairy Sci. 68:2347-2360.

DeRijk, R., F. Berkenbosch. 1994. Suppressive and permissive actions of glucocorticoids: A way to control innate immunity and to facilitate specificity of adaptive immunity? Pages 73-95 in Endocrinology and Metabolism # 7; Bilateral Communication Between the Endocrine and Immune Systems. C. J. Grossman, ed. Springer-Verlag New York Inc., New York, NY.

Detilleux, J. C., M. E. Kehrli, Jr., J. R. Stabel, A. E. Freeman, and D. H. Kelley. 1995. Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet. Immunol. Immunopathol. 44:251-267.

Distelhorst, C. W., B. M. Benutto. 1981. Glucocorticoid receptor content of T lymphocytes: Evidence for heterogeneity. J. Immunol. 126:1630-1634.

Dong, Y., L. Poellinger, J-A. Gustafson, S. Okret. 1988. Regulation of glucocorticoid receptor expression: Evidence for transcriptional and posttranscriptional mechanisms. Mol. Endocrinol. 2:1256-1264.

Filep, J. G., A. Delalandre, Y. Payette, E. Foldes-Filep. 1997. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. Circulation. 96:295-301.

Flaming, K.P., B. L. Goff, D. E. Frank, J. A. Roth. 1994. Pigs are relatively resistant to dexamethasone induced immunosuppression. Comp. Haematol. Int. 4:218-225.

Gagne, D., M. Pons, D. Philibert. 1985. RU 38486: A potent antiglucocorticoid in vitro and in vivo. J. Steroid Biochem. 23:247-251.

Gagne D., M. Pons, A. C. De Paulet. 1986. Analysis of the relation between receptor binding affinity and antagonist efficacy of antiglucocorticoids. J. Steroid Biochem. 25:315-322.

Goff, J. P., M. E. Kehrli, Jr., R. L. Horst. 1989. Periparturient hypocalcaemia in cows: prevention using intramuscular parathyroid hormone. J. Dairy Sci. 72:1182-1187.

Grohn, Y. T., S. W. Eicker, J. A. Hertl. 1995. The association between previous 305-day milk yield and disease in New York State dairy cows. J. Dairy Sci. 78:1693-1702.

Guido, E. C., E. O. Delorme, D. L. Clemm, R. B. Stein, J. Rosen, J. N. Miner. 1996. Determinants of promoter-specific activity by glucocorticoid receptor. Mol. Endocrinol. 10:1178-1190.

Guidry, A. J., M. J. Paape, R. E. Pearson. 1976. Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. Am. J. Vet. Res. 37:1195-1200.

Gwazdauskas, F. C., M. J. Paape, D. A. Peery, M. L. Mc Gilliard. 1980. Plasma glucocorticoid and circulating blood leukocyte responses in cattle after sequential intramuscular injections of ACTH. Am. J. Vet. Res. 41:1052-1056.

Hollenberg, S. M., C. Weinberg, E. S. Ong, G. Cerelli, A. Oro, R. Lebo, E. B. Thompson, M. G. Rosenfeld, R. M. Evans. 1985. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature. 318:635-641.

Janeway, C. A., Jr., P. Travers. 1996. Immunobiology: The immune system in health and disease, 2nd ed. Roberston, M., E. Lawrence, eds. Garland Publishing, Inc., New York, NY.

Junker, K. 1983. Glucocorticoid receptors of human mononuclear leukocytes in vitro. J. Clin. Endocrinol. Metab. 57:506-512.

Kalinyak, J. E., R. I. Dorin, A. R. Hoffman, A. J. Perlman. 1987. Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J. Biol. Chem. 262:10441-10444.

Khosraviani, M., S. L. Davis. 1996. Hormonal regulation of peripheral blood mononuclear cells in sheep. Dom. Anim. Endocrinol. 13:139-150.

Kehrli M. E., J. L. Burton, B. J. Nonnecke, E. K. Lee. 1998. Effects of stress on leukocyte trafficking and immune responses: Implications for vaccination. Pages 61-81 in. *Advances in Veterinary Medicine, Vol 41.* Schultz R. D. ed. Academic Press, New York, NY.

Kehrli, M. E., Jr., B. J. Nonnecke, J. A. Roth. 1989a. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50:207-214.

Kehrli, M. E., Jr., B. J. Nonnecke, J. A. Roth. 1989b. Alterations in bovine lymphocyte function during the periparturient period. Am. J. Vet. Res. 50:215-220.

Kontula, K., T. Paavonen, T. Luukkainen, L. C. Anderson. 1983. Binding of progestins to the glucocorticoid receptor. Biochem. Pharmacol. 32:1511-1518.

Lee, E-K., M. E. Kehrli, Jr. 1998. Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. Am. J. Vet. Res. 59:37-43.

Lipman, M., R. Barr. 1977. Glucocorticoid receptors in purified subpopulations of human peripheral blood lymphocytes. J. Immunol. 118:1977-1981.

Lohuis J. A. C. M., W. Van Leeuwen, J. H. M. Verheijden, A. S. J. P. A. M. Van Miert. A. Brand. 1988. Effect of dexamethasone on experimental *Escherichia coli* mastitis in the cow. J. Dairy Sci. 71:2782-2789.

Mallard, B. A., J. C. Dekkers, M. J. Ireland, K. E. Leslie, S. Sharif, C. Lacey, C. Van Kampen, L. Wagter, B. N. Wilkie. 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J. Dairy Sci. 81:585-595.

Martinez, P. E., M. T. P. Lopez, M. S. Martins, A. P. Marques, Jr., C. E. Salas. 1998. Changes in glucocorticoid receptor levels of bovine lymphocyte and neutrophil during estrous cycle. Reprod. Dom. Anim. 33:61-66.

McCullough, K. C., S. Martinod. Future trends in bovine immunology: Applications for immunomodulators. Western Vet. Conference. February 16, 1987, Las Vegas. p. 30-44.

Miller, A. H., R. L. Spencer, J. Hassett, C. Kim, R. Rhee, D. Ciurea, F. Dhabhar, B. McEwen, M. Stein. 1994. Effects of selective type I and II adrenal steroid agonists on immune cell distribution. Endocrinol. 135:1934-1944.

Molijn, G. J., J. W. Koper, C. J. C. van Uffelen, F. H. de Jong, A. O. Brinkman, H. A. Bruining, S. W. J. Lamberts. 1995. Temperature-induced down-regulation of the glucocorticoid receptor in peripheral blood mononuclear leukocyte in patients with sepsis or septic shock. Clin. Endocr. 43:197-203.

Moreira da Silva, F., C. Burvenich, A. M. Massart Leen, L. Broose. 1998. Assessment of blood neutrophil oxidative burst activity in dairy cows during the period of parturition. Anim. Sci. 67:421-426.

Murakami, T., D. Brandon, D. Rodbard, D. L. Loriaux, M. B. Lipsett. 1979. Glucocorticoid receptor in circulating mononuclear leukocytes. Endocrinol. 104:500-505.

Nagahata, H., S. Makino, H. Takahashi, and H. Noda. 1988. Assessment of neutrophil function in the dairy cow during the perinatal period. J. Vet. Med. 35:747-751.

Nagahata, H., A. Ogawa, Y. Sanada, H. Noda, S. Yamamoto. 1992. Peripartum changes in antibody producing capability of lymphocytes from dairy cows. Vet. Quarterly. 14:39-40.

National Mastitis Council. 1996. Current concepts of bovine mastitis, fourth edition. Madison, WI.

Nonnecke, B. J., J. L. Burton, and M. E. Kehrli. 1997. Associations between function and composition of blood mononuclear leukocyte populations from Holstein bulls treated with dexamethasone. J. Dairy Sci. 80:2403-2410.

Norman, A.W., G. Litwack. 1997. Hormones. Second Edition. Academic Press, California.

Oakley R. H., J. A. Cidlowski. 1993. Homologous down regulation of the glucocorticoid receptor: The molecular machinery. Crit. Rev. Eukaryotic Gene Exp. 3:63-88.

Oakley, R. H., M. Sar, J. A. Cidlowski. 1996. The human glucocorticoid receptor _ isoform. J. Biol. Chem. 271:9550-9559.

Okret, S., L. Poellinger, Y. Dong, J-A. Gustafson. 1986. Down-regulation of glucocorticoid receptor mRNA by the receptor of a specific binding sequence within a receptor cDNA clone. Proc. Natl. Acad. Sci. 83:5899-5903.

Oliver, S. P., L. M. Sordillo. 1988. Udder Health in the periparturient period. J. Dairy Sci. 71:2584-2606.

Paape, M. J., F. C. Gwazdauskas, A. J. Guidry, B. T. Weinland. 1981. Concentrations of corticosteroids, leukocyte, and immunoglobulins in blood and milk after administration of ACTH to lactating dairy cattle: Effects on phagocytosis of *Staphylococcus aureus* by polymorphonuclear leukocytes. Am. J. Vet. Res. 42:2081-2087.

Parillo, J. E., A. S. Fauci. 1979. Mechanisms of glucocortiocid action on immune processes. Annu. Rev. Pharmacol. Toxicol. 19:179-201.

Petrichenko, I. E., Y. A. Shakhov, N. A. Gratsianski, O. I. Aleshin, N. V. Chepurnenko, N. V. Perova. 1989. Number of glucocorticoid receptors in lymphocytes and their sensitivity to hormone action. Experientia 45:1115-1117.

Preisler, M. T., P. S. D. Weber, R. J. Tempelman, R. J. Erskine, H. Hunt, J. L. Burton. 1999. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. Am. J. Vet. Res. (In Press).

Rosewicz S., A. R. McDonald, B. A. Maddux, I. D. Goldfine, R. L. Miesfeld, C. D. Logsdon. 1988. Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. J. Biol. Chem. 263:2581-2584.

Roth, J. A., M. L. Kaeberle. 1982. Effect of glucocorticoids on the bovine immune system. JAVMA. 180:894-901.

Roth, J. A., M. L. Kaeberle. 1981. Effects of in vivo dexamethasone administration on in vitro bovine polymorphonuclear leukocyte function. Inf. Immun. 33:434- 441.

Roth, J. A., M. L. Kaeberle, W. H. Hsu. 1982. Effects of adrenocorticotropin administration on bovine polymorphonuclear leukocyte function and lymphocyte blastogenesis. Am. J. Vet. Res. 43:412-416.

Rupprecht R., J. M. H. M. Reul, B. van Steensel, D. Spengler, M. Soder, B. Berning, F. Holsboer, K. Damm. 1993. Pharmacological and functional characterization of human mineralcorticoid and glucocorticoid receptor ligands. Eur. J. Pharm. 247:145-154.

SAS/STAT Software: Changes and Enhancement through release 6.11. 1996. SAS Inst., Inc., Cary, NC.

SAS Procedures Guide, Version 6, Third Edition. 1990. SAS Inst., Inc., Cary, NC.

Saad, A. M., C. Concha, G. Astrom. 1989. Alterations in neutrophil phagocytosis and lymphocyte blastogenesis in dairy cows around parturition. Zentralbl Veterinarmed 365:337-345.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci. 51: 660-665.

Schlecte, J. A., B. H. Ginsberg, B. M. Sherman. 1982. Regulation of the glucocorticoid receptor in human lymphocytes. J. Steroid Biochem. 16:69-74.

Shipman, G. F., C D. Bloomfield, K. J. Gajil-Peczalska, A. U. Munck, K. A. Smith. 1983. Glucocorticoids and lymphocytes. III. Effects of glucocorticoid administration on lymphocyte glucocorticoid receptors. Blood. 61:1086-1090.

Sigal, G. A., D. A. Maria, M. L. H. Katayama, B. L. Wajchenberg, M. Brentani. 1993. Glucocorticoid receptors in mononuclear cells of patients with sepsis. Scand. J. Infect. Dis. 25:245-248.

Smith V. G., L. A. Edgerton, H. D. Hafs, E. M. Convey. 1973. Bovine serum estrogens, progestins and glucocorticoids during late pregnancy, parturition, and early lactation. J. Anim. Sci. 36:391-396.

Staples, L. D., R. M. Binns, R. B. Heap. 1983. Influence of certain steroids on lymphocyte transformation in sheep and goats studied in vitro. J. Endocr. 98:53-69.

Stossel, T. P. 1974. Phagocytosis. N. Engl. J. Med. 290:717-723, 774-780, 833-839.

Sutherland R. D., A. Keating, R. Nayar, S. Anania, A. K. Stewart. 1994. Sensitive detection and enumeration of $CD34^{+}$ cells in peripheral cord blood by flow cytometry. Exp. Hematol. 22:1003-1010.

Tronche F., C. Kellendonk, H. M. Reichardt, G. Schutz. 1998. Genetic dissection of glucocorticoid receptor function in mice. Curr. Opinions Gen. Dev. 8:532-538.

Trowbridge I. S., M. L. Thomas. 1994. CD45: An emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annu. Rev. Immunol. 12:85-116.

Van Kampen, C., B. A. Mallard. 1997. Effects of peripartum stress and health on circulating bovine lymphocyte subsets. Vet. Immuno. Immunopathol. 59:79-91.

Wesley I. V., J. H. Bryner, M. J. Van Der Maaten, M. Kehrli. 1989. Effects of dexamethasone on shedding of *Listeria monocytogenes* in dairy cattle. Am. J. Vet. Res. 50:2009-2013.

Weyts, F. A. A., B. M. L. Verburg-van Kemenade, G. Flik. 1998. Characterization of glucocorticoid receptors in peripheral blood leukocytes of carp, *Cyprinus carpio* L. Gen. Comp. Endocr. 111:1-8.

Wilckens, T. , R. DeRijk. 1997. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol. Today. 18:418-423.