The effects of stress on bovine neutrophil adhesion molecules and fecal bacterial coliform shedding

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The effects of stress on bovine neutrophil adhesion molecules and fecal bacterial coliform shedding

by

Lisa Suzanne Pelan-Mattocks

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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ABSTRACT

Stress in cattle, including nutritional deprivation, parturition, drugs, and bacterial and viral infections, decreases neutrophil function and trafficking and is associated with increased susceptibility to infectious disease. Neutrophils are vital to the inflammatory response, and impaired neutrophil function is associated with predisposition to new intramammary infection, respiratory disease, and other illnesses. Periparturient cows have a high incidence of coliform mastitis and newborn calves experience diarrhea caused by coliform bacteria. In our first study we investigated whether fecal coliform bacteria shedding from cows increases around calving time, thereby increasing the number of coliforms in the environment of the cow and calf. Our data demonstrate considerable variability in shedding of coliform bacteria in feces. All cows studied, experienced a $10^4-10^7$ cfu/gram of feces increase in fecal coliform bacteria shedding within 12 days of parturition. This suggests increased shedding of fecal coliform bacteria near parturition may contribute to the increased rate of coliform infections in periparturient cows and calves. Immunosuppression induced by anti-inflammatory drugs can exacerbate subclinical infections into clinical disease. In our second study, we assessed and compared the effects of a single pharmacological dose of selected non steroidal anti-inflammatory drugs (NSAIDs) on leukocyte adhesion molecule expression and neutrophil functions with that of a glucocorticoid, dexamethasone. NSAIDs are commonly used for treatment of various inflammatory conditions accompanied by endotoxemia or pain. If NSAIDs have negative effects on leukocyte function, their extended use in diseased animals could be contraindicated. Our results indicate that aspirin, ibuprofen, flunixin meglumine, and phenylbutazone have no negative effects on neutrophil adhesion molecule expression or other neutrophil functions tested. In our final study, we evaluated an efficient and reliable method to determine bovine blood leukograms. that accurately differentiates bovine lymphocytes from monocytes. A flow cytometric method was adapted to assess differential leukocyte counts on bovine blood, using CD45 (leukocyte
common antigen). Fast preparation and analysis times allow for efficient and reliable examination of a large number of samples, and the laborious task of reading numerous slides is eliminated.
GENERAL INTRODUCTION

Thesis Organization

The following dissertation is organized into a general introduction, three journal papers, an appendix, and a general summary. The general introduction consists of a review of the literature of leukocyte trafficking and the importance of neutrophils in the immune response, and the effects that various stressors (i.e. parturition, feed deprivation, and administration of glucocorticoids and non-steroidal anti-inflammatory drugs) may have on bovine neutrophil function and subsequent susceptibility to disease. The appendix contains an abstract and graphs from a preliminary project (work unpublished) assessing various parameters of fasting stress in calves.

Introduction

Neutrophils are a vital component in the inflammatory response to tissue injury and microbial infection. Neutrophils are a leukocyte subpopulation of somewhat unsophisticated effector cells. They represent important components of both native and acquired immunity in mammals, and are the most important of the phagocytic cells that defend the host against acute bacterial infection. Neutrophils have a particularly important role in protecting skin, and the mucous membrane linings of the respiratory and gastrointestinal tracts. As such, they provide the first line of defense against microbial invaders. Therefore it is not surprising when bovine neutrophil function is severely compromised that the animal is predisposed to cutaneous and mucous membrane infections. Nutritional deficiencies, hormones, drugs, and bacterial and viral infections have been shown to hamper neutrophil function in cattle. Stress, including parturition, feed deprivation, and glucocorticoid administration, have been shown to have profound effects on neutrophil trafficking and function and have been associated with increased susceptibility to infectious disease.
Literature Review

Leukocyte trafficking

An integral component in host defense is the ability of leukocytes to be effectively recruited into peripheral tissues following invasion by microorganisms or tissue injury. Trafficking enables leukocytes to survey tissue for foreign material, and is an important mechanism for rapid leukocyte accumulation at sites of infection or injury. Most mature lymphocytes recirculate continuously, patrolling the body by recirculating from blood, through tissue, into the lymphatic system, and back into the blood, as often as one to two times per day. The interaction of different populations of circulating leukocytes with postcapillary venular endothelial cells is essential for leukocyte emigration into tissue. Lymphocytes tend to recirculate or “home” through the same environment based on prior exposure to foreign material. Trafficking is not a random process. The interaction between leukocytes and endothelial cells is a dynamic process involving both cell types with specialized adhesion pathways. Previous theories suggesting that the endothelium is an inert lining for blood vessels are no longer valid. Human, rodent, and porcine lymphocytes extravasate into lymph nodes through specialized paracortical postcapillary venules that are lined with high endothelium, thus called high endothelial venules (HEV). Leukocyte extravasation is a multi-step process involving: initial interaction of leukocytes with activated endothelium (rolling), leukocyte activation with firm adhesion to endothelial cells, and extravasation into the surrounding tissue.

A consensus model of recruitment has been established. In this model, the interaction between leukocytes and endothelium is the critical factor, and it is a dynamic process. Within this model of extravasation are concepts that were discovered over the course of the last century. In 1889, Cohnheim, using intravital microscopy, observed leukocytes interacting with the vessel wall by rolling along the endothelium within minutes after injury to adjacent tissue. In observing the progression of an inflammatory reaction, he
further observed that the endothelium becomes lined with leukocytes, and their rolling decreases in velocity and is interrupted by transient halts until they come to a firm stop. A transmigration process follows by which the cell flattens and a pseudopod grows in size until the entire cell body has emerged through a narrow gap between endothelial cells. Cohnheim postulated molecular changes in the vessel endothelium which control the process of inflammation.

In 1962, hydrodynamic studies of particles in suspension showed that in Poiseuille flow, the larger particles are forced to the center of the stream, and this effect is more pronounced as shear forces increase. (This was called Poiseuille flow after Jean Poiseuille who related the volume flow rate with the viscosity of a fluid, the radius and length of an object, and pressures at the ends of the object by formulating an equation for laminar, non-pulsatile flow through a uniform pipe. The use of Poiseuille’s Law on the human circulatory system is highly suspect.) This effect has been shown to be true for blood cells both in vivo and in vitro; the larger leukocytes are forced to the center of the stream in normal flow. In inflammation two events occur. First, vessels dilate thus slowing flow rate. secondly, capillary permeability is increased due to retraction of the endothelial cells, allowing for soluble mediators of immunity to pass to infection sites. These factors cause leukocytes to be displaced to the vessel wall. Because fluid velocity is highest in the center of the vessel and not near the wall, cells will tumble. The velocity at which these cells tumble is much faster than observed for rolling cells in an inflammatory reaction, suggesting that additional adhesive interactions occur between the leukocyte and vessel wall in the event of inflammation.

The molecular basis of leukocyte rolling still is not fully elucidated. However, it has been determined that the recruitment of leukocytes to sites of acute inflammation involves the combined action of multiple families of adhesion molecules, chemoattractants, and cytokines. Several adhesion molecules are involved in the process of adhesion and migration of
leukocytes through vascular endothelium at sites of inflammation. Selectivity in the process is determined by the diversity of these molecules and their ability of mediating each step.

**Adhesion molecules control cell attachment and transendothelial migration**

As leukocytes circulate throughout the body surveying for foreign material, they adhere transiently to one another, to other cell types (such as vascular endothelial cells), and after extravasation, to extracellular matrix proteins. This adhesion ability is mediated by families of specific cell surface adhesion molecules. Three groups have been characterized: (1) the integrin family, which is important in dynamic regulation of adhesion and migration. (2) the selectins, which are prominent in initial contact of leukocytes with vascular endothelium, and (3) immunoglobulin superfamily molecules, which include various cellular adhesion molecules (CAMs). Adhesion not only allows for the recirculating and homing of lymphocytes into lymphoid organs and the leukocyte migration into extravascular tissues, it is also required for leukocyte-mediated cytotoxicity, phagocytosis, chemotaxis, and induction of lymphocyte proliferation and maturation. However, adhesion is not always beneficial; it can also be detrimental in various immune and inflammatory responses. Excessive accumulation of leukocytes can lead to inflammatory disease and tissue injury.

**Selectins**

Selectins mediate a function unique to the vasculature: they allow for the initial contact and loose attachment, or “tethering”, of moving leukocytes to the vessel wall through “sticky” carbohydrate ligands that permit leukocytes to roll along on endothelia of postcapillary venules. They are involved very early in the cascade of molecular events that ultimately allows leukocytes to leave the blood stream.

Selectins are heavily glycosylated, single chain integral membrane proteins. The selectins consist of only 3 known members: CD62L (L-selectin, lymphocyte homing receptor.
LECAM-1, LAM-1, LECAM, gp90Mel, Mel-14), CD62E (E-selectin, ELAM-1), and CD62P (P-selectin, GMP-140, PADGEM). CD62L is found on the tips of leukocyte microvilli and is primarily required for lymphocyte binding to endothelium in peripheral lymph nodes during lymphocyte recirculation and also for neutrophil emigration from post capillary venules at sites of inflammation. It is constitutively expressed, and is rapidly cleaved upon leukocyte activation.\textsuperscript{32} CD62L interaction with its ligand is non-reversible, enabled by proteolytic cleavage of CD62L near the cell membrane upon cross-linking of multiple CD62L molecules. Although, multivalent CD62L crosslinking alone cannot cause complete leukocyte arrest, CD62L is vital for recruitment of circulating neutrophils into inflamed tissue and lymphocyte homing into lymph nodes.\textsuperscript{13,33}

CD62P is stored in granules of platelets and Weibel-Palade bodies of endothelial cells.\textsuperscript{34,35} Expression is upregulated at the endothelial or platelet cell surface by the effects of thrombin, histamine, and peroxides. Expression is short lived, and therefore this molecule likely binds its ligand on circulating leukocytes to enable an early step in leukocyte adhesion to endothelium at sites of inflammation. In contrast, CD62E is neither synthesized constitutively nor stored within intracellular granules.\textsuperscript{36} CD62E is synthesized by endothelial cells in response to inflammatory cytokines such as IL-1 and TNF.

All ligands for each selectin have not been clearly elucidated, although it is believed that ligands for selectins are diverse and complex macromolecules that share common types of anionic carbohydrates.\textsuperscript{37} The importance of selectins is exemplified by a human genetic disease, termed leukocyte adhesion deficiency type 2 (LAD 2), in which a fucosylation failure reduces expression of all fucosylated lactosamines and selectin-mediated binding is severely diminished.\textsuperscript{38} The patients of LAD 2 show a high blood neutrophil count, marked defects in neutrophil motility, recurrent pneumonia, and bacterial infection, which emphasize the importance of the adhesive interactions mediated by selectins during the inflammatory response.\textsuperscript{38}
Integrins

Integrins are involved in numerous biological adhesion functions including embryonic development, platelet aggregation, tissue maintenance and repair, and most importantly, integrins are the major family of cell surface receptors that mediate leukocyte recruitment and extravasation.\(^3\) The term “integrin” was originally used to describe membrane receptors which integrate the extracellular environment with the intracellular cytoskeleton.\(^3\) However the name was found to be even more appropriate when an additional function was discovered three years later. Integrins help to integrate many of the diverse signals that impinge on cells; in doing so, they determine a cell’s fate.

Integrins are heterodimers consisting of noncovalently associated α and β transmembrane subunits selected from among 16 α variants and 8 β variants. Certain α chains can combine with more than one β chain thus yielding at least 20 different integrins. Furthermore, most integrins recognize and bind to more than one target ligand. Leukocytes express at least 13 of these integrin heterodimers.\(^4\) One subfamily in particular, the β₂ integrins, play an important role in the function of leukocyte extravasation.

The β₂ integrins, termed the CD₁₁/CD₁₈ family, have also been known as LeuCAMs, or “leukocyte” antigens. This group consists of 4 leukocyte adhesion receptors, CD₁₁α/CD₁₈ (LFA-1), CD₁₁b/CD₁₈ (MAC-1, Mo-1, or CR3), CD₁₁c/CD₁₈ (p150,95 or CR4), and CD₁₁d/CD₁₈. Each of these molecules contains a noncovalently associated “α” (CD₁₁) and a “β” (CD₁₈) subunit. As their names imply, they share an identical β subunit. CD₁₈, and are distinguished by their respective α subunits designated as CD₁₁a, CD₁₁b, CD₁₁c, and CD₁₁d. All but CD₁₁d have been found in the bovine. The β₂ integrins are primarily involved in leukocyte-endothelial cell contact or leukocyte-leukocyte interactions.\(^4\)

CD₁₁α/CD₁₈ is involved in T cell, natural killer cell, and antibody dependent cytotoxicity as well as T helper cell functions, and is also important in monocyte-endothelial cell interaction.\(^4\) Ligands for CD₁₁α/CD₁₈, include ICAM-1 (intercellular adhesion molecule-
1) and ICAM-2, members of the Ig superfamily, which are expressed on many different cell types. CD11a/CD18 is expressed on all leukocytes, however, it is the only $\beta_2$ integrin expressed on T and B lymphocytes.\(^4^3\) CD11a/CD18 contributes to effective diapedesis of lymphocytes, although it is not necessarily required for egress.

CD11b/CD18 and CD11c/CD18 are found predominantly on granulocytes, monocytes, and natural killer cells. They are stored in secondary and tertiary granules, and a remarkable increase in surface expression of these molecules occurs following cellular activation due to translocation of intracellular granules to the cell surface.\(^4^3\) CD11b/CD18 has a critical role in the complement cascade as it is complement receptor 3 (CR3) which binds to the iC3b product of activated complement. Additionally, CD11b/CD18 is particularly important in neutrophil-endothelial cell binding. In contrast to CD11a/CD18 and CD11b/CD18, cellular ligands for CD11c/CD18 are still unknown. CD11c/CD18 is also known as complement receptor 4 (CR4).

The importance of $\beta_2$ integrins is emphasized by an autosomal recessive disease seen in humans, Irish setter dogs, and Holstein-Friesian cattle termed leukocyte adhesion deficiency (LAD) Type I, characterized by unthriftiness, soft tissue infections, severely impaired pus formation, constant leukocytosis, and a wide spectrum of deficits in adhesion dependent functions of granulocytes, monocytes, and lymphocytes.\(^4^4^4^8\)

**Immunoglobulin Superfamily**

Members of the immunoglobulin superfamily play an important role in further strengthening adhesive interactions between leukocytes and endothelial cells of the postcapillary venules. These proteins, containing immunoglobulin-like domain structures, bind to integrins and selectins expressed on leukocytes. This superfamily includes the cellular adhesion molecules (CAMs): ICAM-1 (intercellular CAM-1), ICAM-2, VCAM-1 (vascular
CAM-1), PECAM-1 (platelet endothelial CAM-1), and MAdCAM-1 (mucosal adhesion CAM-1). All members of this family are expressed, or are inducible, on vascular endothelium. This family includes proteins of endothelial cells that bind to the β2-integrins expressed on leukocytes. ICAM-1 is involved in binding CD11a/CD18, and CD11b/CD18, depending on which domains of the ICAM molecule are used. ICAM-1 is expressed at low levels on resting endothelial cells, but its expression is upregulated by various cytokines. Interestingly, ICAM-1 is a ligand for the major group of rhinovirus serotypes that cause the common cold. ICAM-2 is constitutively expressed on leukocytes and at a high level on resting endothelial cells, but its expression is not heightened by activation.

**Effects of anti-inflammatory compounds on trafficking**

Although neutrophils are the most important phagocytic cells that defend the host against bacterial infection, neutrophils have been implicated as producing inappropriate tissue injury during inflammation. Several strategies of therapeutic modulation of neutrophil inflammatory function have aided in circumventing the destructive effects of activated neutrophils. Various points during the inflammatory response, including neutrophil recruitment, priming, and activation leading to hyperadherence, diapedesis, and generation of tissue destructive factors can be blocked with anti-inflammatory agents. However, some of these intervention tactics actually cause a suppression of the immune system, through inhibiting neutrophil function. This section will focus on two classes of anti-inflammatory agents, glucocorticoids and nonsteroidal anti-inflammatory drugs, and their current status as anti-inflammatory mediators.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are defined as a heterogeneous group of anionic, planar, lipophilic compounds frequently prescribed for their analgesic, antipyretic, and anti-inflammatory properties. These anti-inflammatory agents are commonly used for treatment of various disorders of cattle including diarrhea, septicemia, pneumonia, acute
mastitis, lameness and other conditions accompanied by endotoxemia or pain.\textsuperscript{58-62} Although several NSAIDs, including flunixin meglumine, phenylbutazone, dipyrene, ibuprofen and aspirin are available for veterinary use, only aspirin and ketoprofen are approved for the use in treatment of food producing animals.\textsuperscript{63} Appropriate dosage, treatment intervals, and withdrawal guidelines for milk, meat, and eggs are not readily available and in some cases are undefined. Despite this lack of knowledge, veterinarians use various NSAIDs by the flexibility given in “extra-label” prescriptions.

Inhibition of cyclooxygenase, the enzyme responsible for the biosynthesis of prostaglandins and certain related autacoids, is generally thought to be the major mechanism of action of NSAIDs.\textsuperscript{64} However, this mechanism does not fully explain all effects of NSAIDs.\textsuperscript{65,66} Some NSAIDs have been suggested to also interfere with signal transduction, thus affecting neutrophil function, including adhesion interactions.\textsuperscript{67-69} As described earlier, a major aspect of neutrophil function is adhesion. Adhesion among neutrophils and adhesion of neutrophils to the blood vessel wall are imperative for neutrophils to egress from the stream of blood flow into tissue.

Glucocorticoids are known to have anti-inflammatory effects. Glucocorticoids are effective anti-inflammatory agents partly because of their suppressive effects on neutrophil function. They inhibit the phospholipase A\textsubscript{2} enzyme which inhibits release of arachadonic acid from cell membrane phospholipids and consequently inhibits arachidonic acid metabolism. It is well documented that in vivo glucocorticoid treatment results in the inhibition of neutrophil function tested ex vivo.\textsuperscript{70-72} Roth \textit{et al} have reported that dexamethasone, a potent synthetic glucocorticoid, when administered to cattle significantly decreases neutrophil iodination, antibody-dependent cell-mediated cytotoxicity and oxidative metabolism, and enhances neutrophil random migration.\textsuperscript{73} Administration of dexamethasone to cattle also causing transient shedding of CD62L and decreased expression of CD18.\textsuperscript{74} The production of interferon-\(\gamma\) (IFN-\(\gamma\)) and IgM is also inhibited by dexamethasone administration.\textsuperscript{75,76}
Glucocorticoid administration will also suppress or delay some antigen-specific immune responses to vaccines.77 Other mechanisms have also been reported to further explain some of glucocorticoids anti-inflammatory properties. Anti-inflammatory effects of glucocorticoids may be partially mediated by factors released by monocytes78 and may also work by mechanisms independent of arachidonic acid metabolism.79 Prolonged administration of glucocorticoids is contraindicated in animals with underlying chronic infections due to the immunosuppressive effects on leukocytes which can cause subclinical infections to convert into acute clinical disease.80,81 If NSAIDs were also to have immunosuppressive effects on leukocyte functions, their extended use in diseased animals could be contraindicated.

Interference with signal transduction and adhesion molecule interactions has been reported for some NSAIDs, although murine and human in vitro studies have been inconsistent. Diaz-Gonzalez et al suggest that NSAIDs’ function is dependent upon shedding of L-selectin while Andrews et al suggest that NSAIDs’ mechanism of action is dependent upon their ability to upregulate ICAM-1.82,83 Fiorucci et al report NSAIDs upregulate β2-integrin expression on human neutrophils, while Cronstein et al state that all NSAIDs inhibit neutrophil aggregation without affecting β2-integrins.84,85 Others have published that NSAIDs up-regulate tumor necrosis factor (TNF), IFN-γ, and interleukin 2 (IL-2) production at both the mRNA and protein levels, and down-regulate interleukin 4 (IL-4) mRNA expression.86 It is well recognized that NSAIDs exert biologic effects other than the inhibition of prostaglandin synthesis. NSAIDs inhibit a variety of membrane-associated processes and enzymes including NADPH oxidase, phospholipase C, 12-hydroperoxyeicosatetraenoic acid peroxidase and transmembrane anion transport.65 Moreover, they inhibit the aggregation of stimulated human neutrophils induced by FMLP (bovine neutrophils cannot be stimulated by FMLP).68 Therefore, the claims for the potential anti-inflammatory properties of NSAIDs are not always restricted to their inhibition of cyclooxygenase. Depending on the compound (each with
different half-lives), species studied, and type of study, one can conclude that there are multiple effects of the NSAIDs and that much still needs to be elucidated.

A national survey conducted in 1992, showed that 93% of veterinarians responding used NSAIDs in treating food-producing animals. Over half of these reported prescribing NSAIDs more than once a week. Flunixin meglumine, dipyrone, aspirin and phenylbutazone were the most frequently used NSAIDs. Although the mechanism of action of NSAIDs is not fully elucidated, it seems their use in cattle may be a safe and effective alternative to glucocorticoids. Anderson et al have shown that multiple doses of flunixin meglumine improve clinical manifestations (by reducing rectal temperature, depression, and clinical signs of quarter inflammation) of acute mastitis induced by intramammary inoculation of Escherichia coli endotoxin and that adverse effects were not observed. Similarly, it has been shown that ibuprofen prevents endotoxin-induced pyrexia in cattle and that ibuprofen provides some beneficial treatment in reducing heart and respiratory rates in similar cases. Importantly, no impairment of neutrophil phagocytosis nor other neutrophil function were found in cows given flunixin meglumine or ibuprofen.

**Effects of natural stress on leukocyte trafficking and function**

In the livestock industry, stress is an important component of disease, and subsequently affects cost. Stress can be broadly defined as events in which homeostasis of the animal and or its environment are disturbed or threatened. In this situation, an animal is forced to respond in order to maintain equilibrium. Although stress reactions are organized to protect the homeostatic state of animals, they contain elements that may either enhance or diminish susceptibility to disease processes; in many instances however, stress reactions themselves may induce pathologic change. All stress is not harmful to an animal; without stress, animals could not survive. Release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which induces synthesis and secretion of glucocorticoids from the adrenal cortex, is one
example of the host response to stress. Additionally, this release of ACTH occurs during a response to infection and is likely involved with the natural down-regulation of the innate immune response.\textsuperscript{87} This down-regulation has also been associated with hormones such as cortisol, endogenous opiates, and catecholamines.\textsuperscript{88} Cortisol levels have been shown to rise in events such as shipping, weaning, parturition, administration of glucocorticoids, and estrous.\textsuperscript{89-93}

The best examples of alterations in leukocyte trafficking and immune function associated with stress are studies of pregnancy and parturition of many species. Periparturient and neonatal immunosuppression is suggested by the increased incidence and susceptibility of cows and calves to bacterial and viral infections during this period.\textsuperscript{94,95} Hormonal changes occur during the periparturient period\textsuperscript{96} likely contribute to hampering proper immune function. Increased plasma concentration of the endogenous opioids, \textbeta-endorphin and metenkephalin, during the periparturient period in cows may also reduce immune function.\textsuperscript{88} Plasma concentration of these opioids peaks at parturition and cows experiencing dystocia have significantly elevated concentrations of \textbeta-endorphin several hours postpartum compared to normal cows. Immunologic disturbances in cellular and humoral components of immune responses have been documented in cattle during the periparturient period (neutrophil chemokinesis, respiratory burst, phagocytosis, lymphocyte blastogenesis, and serum concentration of immunoglobulins) and related to the marked reduction in the ability of dairy cattle to respond to invasive microorganisms.\textsuperscript{2,4,5,97-99}

Pregnancy has been suggested to result in suppression of cell-mediated immune function and enhancement of humoral immunity. During the periparturient period, animals undergo marked physiologic changes that might cause suppression of host defense mechanisms and an increase in susceptibility to uterine and mammary gland infection.\textsuperscript{100,101} As pregnancy progresses, increases in estrogen and progesterone occur, which are known suppressors of cell-mediated immune responses and enhancers of humoral responses in
mice. Cytokine production by bovine leukocytes is also disturbed during the periparturient period in that IFN-γ and IL-2 production are reduced, suggesting suppression of the Th1 immune response. Furthermore, it has been suggested that changes in cytokine production might contribute to the increased incidence of disease in periparturient cows. Moreover, impairment of the capacity of B cells from periparturient cows to secrete IgM has been reported which may be due to impaired IFN-γ and IL-2 production.

Leukocyte trafficking patterns are also altered in periparturient cows. A decline in CD18 and CD62L expression on both cow and calf neutrophils from calving to 15 hours postpartum occurs, and may be correlated with elevated plasma cortisol concentrations at that time. Low expression of CD62L on neutrophils from cows, and lowered expression of both CD62L and CD18 on calf neutrophils for several days postpartum may result in impaired inflammation. Leukocytosis due to a neutrophilia co-incident with decreased expression of CD62L on neutrophils at parturition may cause diminished egress of neutrophils from blood into tissues of cows and calves. Additionally, the percentage of T cells declines, along with the proportion of CD4+ cells in blood and mammary parenchyma.

Other equally stressful situations cause increased levels of cortisol and alteration in immune function in cattle. Road transport increases cortisol release, susceptibility to disease, and affects reproduction in cows. Conditions such as weaning, handling, castration and dehorning, forced exercise, and situations where the animal encounters acute pain also increase plasma cortisol lending to increased disease susceptibility.

**Fasting stress effects on health and fecal shedding of coliform bacteria**

The stress of hunger initiates feeding, yet, the stress of removing food causes an increased stress situation. Fasting stress not only causes increases in plasma cortisol levels but also alters other hormone levels.
There is evidence that animals deprived of feed may exhibit increased fecal coliform bacteria shedding.\textsuperscript{115,116} Brownlee and Grau in 1967 demonstrated that the incidence and quantity of \textit{E. coli} and \textit{Salmonella} shed in feces of cattle and sheep dramatically increases with feed intake restriction. Normally, the rumen represents a hostile environment for enteric bacteria, such as \textit{E. coli}, with only $10^3$-$10^6$ viable \textit{E. coli}/ml existing among the $10^9$-$10^{10}$ cells/ml of total rumen bacteria. \textit{Salmonella sp} and \textit{E. coli} are particularly sensitive to the pH and volatile fatty acid concentration of the rumen.\textsuperscript{116,117} In a well-fed animal, growth inhibition of \textit{E. coli} and \textit{Salmonella sp} is greatest at pH < 6.5 and volatile fatty acid concentrations >100mM.\textsuperscript{118} However, during periods of feed restriction, pH levels in the rumen can exceed 7.0 and volatile fatty acid concentrations decline (<50mM),\textsuperscript{119,120} both of which allow for \textit{E. coli} survival and growth. It has been reported that most periparturient cows exhibit a 15-30\% decline in dry matter feed intake around parturition.\textsuperscript{121} An increase in fecal bacterial shedding would yield an increased environmental bacterial exposure to the cow concurrent with periparturient immunosuppression, increasing her propensity for disease.

\textbf{Conclusion}

Stress in cattle, including parturition, feed deprivation, and glucocorticoid administration, have profound effects on decreased neutrophil trafficking and are associated with increased susceptibility to infectious disease. Neutrophils are a vital component in the inflammatory response to tissue injury \textit{and} microbial infection, therefore it is not surprising when neutrophil function is severely compromised that the host is predisposed to infection. During the periparturient period it has been reported that various alterations in immune function occur, rendering the animal more susceptible to infection and disease. Additionally, use of some anti-inflammatory agents in animals might further alter trafficking, and perhaps affect the intestinal microflora by changing the balance struck between the microflora and optimal immune function.
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VARIABLE SHEDDING OF FECAL COLIFORM BACTERIA FROM PERIPARTURIENT DAIRY COWS.

A paper submitted to the American Journal of Veterinary Research
L.S. Pelan-Mattocks, M.E. Kehrli, Jr., T.A. Casey, and J.P. Goff

Structured Abstract

Objective- To investigate whether fecal coliform bacteria shedding in dairy cows increases during the periparturient period, and to assess any correlation between feed intake changes and fluctuations in numbers of fecal coliforms.

Animals- 12 healthy, Holstein cows (study 1, n= 5; study 2, n= 7).

Procedure- Two separate studies were conducted. Fecal samples were obtained on a regular basis from approximately 4-6 weeks prepartum through 3 days (study 1) or 2 weeks (study 2) postpartum. Samples were cultured for total fecal coliform bacteria. Additionally, in study 2, daily feed intake was monitored.

Results- During the first 2 weeks of sampling 11 of 12 cows demonstrated low fecal coliform bacteria shedding levels with minimal fluctuations (± 2 log10/gm feces). Coliforms were completely undetectable in 4-8% of the samples taken from 7 of the 12 cows. 90% of the negative samples were taken 33 to 15 days prior to calving. All cows experienced an increase of 10^1-10^7 cfu/gm of feces near parturition. Fecal coliform shedding peaked within 7 days of parturition in 9 of 12 cows, and within 12 days in the remaining 3 cows. No association with changes in feed intake was observed.

Conclusions- Periparturient cows experience large increases in fecal coliform shedding which may contribute to the high incidence of coliform mastitis, as well neonatal diarrhea. Periparturient cows do not continually shed high levels of coliforms in their feces nor are coliforms always detectable by conventional culture methods. Changes in fecal coliform shedding does not seem to correlate with changes in dry matter intake.
Introduction

Intramammary infections (IMI) can be caused by teat end exposure to opportunistic pathogens existing in the environment of the cow. These pathogens include coliform bacteria and streptococci other than Streptococcus agalactiae, and are often found in organic bedding material, manure-covered pens and yards, heavily contaminated water, and wet or damp floor areas. Although postmilking teat disinfection, proper maintenance of milking equipment, and dry cow therapy are effective in reducing the numbers of contagious pathogens, such methods are usually ineffective at reducing new IMI caused by environmental pathogens.

Clinical mastitis caused by environmental pathogens, as opposed to subclinical infection by contagious pathogens, is the number one mastitis problem for well-managed dairies with low somatic cell counts, and concern continues to grow that prevalence of IMI caused by environmental pathogens may be increasing. The increased prevalence may be due to the decrease in percent quarters infected with contagious mastitis, and the trend toward more confinement housing, increasing herd size and decreasing cow time on pasture. As available housing area per cow decreases, the incidence of coliform mastitis increases. Coliforms, environmental streptococci and unknown causes account for greater than 80% of clinical mastitis on well-managed dairies. Despite this high percentage, detection of these pathogens can be difficult because these IMIs are often of short duration.

Periparturient cows have a high incidence of new IMI and clinical mastitis caused by coliform bacteria. During lactation, greater than 75% of Escherichia coli IMI have been associated with calving and the first 90 days into the postpartum period. Periparturient immunosuppression is suggested by the increased incidence and susceptibility of cows to bacterial and viral infections during this period. Immunologic disturbances in cellular and humoral components of immune responses have been documented in cattle during the periparturient period and have been implied to result in a marked reduction in the ability of
dairy cattle to respond to infectious diseases. The rates of new IMI are 2-12 times higher during the first and last 2 weeks of a 60 day nonlactating period than any other time during the production cycle of dairy cows.

Other factors affecting susceptibility to IMI are parity, bedding, and season. Multiparous cows have three times the rate of coliform IMI as do primiparous cows. Secondly, the number of bacteria in bedding is directly correlated with environmental pathogen IMI rates. Organic bedding materials yield significantly higher bacterial levels than inorganic bedding. High levels of gram-negative bacteria in bedding correlate with high rates of clinical mastitis.

Because periparturient cows have a high incidence of coliform mastitis and newborn calves experience diarrhea caused by coliform bacteria, we investigated whether fecal coliform bacteria shedding from cows may increase around calving time thereby increasing the number of these bacteria in the environment of the cow and calf. The objectives of the two studies reported here were, 1) to determine whether coliform bacterial numbers change in feces during the periparturient period and, 2) whether fluctuations in fecal coliform bacteria shedding were associated with changes in dry matter intake.

Materials and Methods

Experimental Design- Two studies were conducted to monitor shedding of coliform bacteria in feces of twelve periparturient cows. In the first study, multiparous periparturient cows were monitored during the summer months (June 1995 through September 1995). The second study focused on shedding of coliform bacteria in feces of periparturient cows during the fall and winter months (October 1995 through February 1996). In both studies, fecal samples were obtained per rectal palpation at approximately 0700 hrs from 3 days/wk up to 7 days/wk as calving date approached. Within one hour of collection, 3-5 g of wet weight fecal samples were diluted [1 gm feces/ 5 ml phosphate buffered saline (PBS. 15 mM KH$_2$PO$_4$, 8
mM Na$_2$HPO$_4$, 137 mM NaCl, 2.6 mM KCl, pH 7.4)]\), and vortexed for 1 minute. Serial dilutions in PBS were made out to at least $5 \times 10^5$ and up to $5 \times 10^7$; and 0.1 ml aliquots were plated on MacConkey's agar and sorbitol MacConkey's agar (SMAC. Sorbitol MacConkey Agar No.3: OXOID, Unipath Ltd., England) and incubated at 37°C for approximately 20 hrs.$^{21,22}$ Only those colonies characteristic of coliforms (lactose fermentors on MacConkey's agar) were counted.$^{23}$

In the first study, samples were obtained from 5 pregnant Holstein cows approximately 4-6 weeks prepartum through 3 days postpartum. Sampling for \textit{E.coli} O157:H7 was also done on days following a day of detecting elevated total fecal coliform shedding. \textit{Escherichia coli} O157:H7 positive colonies would appear white (i.e. do not ferment sorbitol) on SMAC cultures.$^{23}$

In the second study, samples were obtained from 7 pregnant Holstein cows beginning approximately 4-6 weeks prepartum and continuing through 2 weeks postpartum and processed as above. Two, nutritionally-balanced rations were designed for the end of gestation and the early lactating period,$^{24}$ and daily feed intake was recorded.

Data Analysis- Using linear regression, correlation coefficients between feed intake changes and fluctuations in fecal coliform bacteria shedding were determined using Statistical Analysis System®, SAS Institute, Inc. The program PROC CORR was used to determine these correlation coefficients.

Results

\textit{Study 1: Bacterial shedding in 5 periparturient cows}- All 5 periparturient cows exhibited an increase of $10^4$-$10^7$ cfu/g feces near parturition, with 4 of the 5 cows having peak increases within 7 days of parturition. In each cow, shedding of fecal coliform bacteria was relatively constant (0-2 log10 changes) approximately 34 to 25 days before parturition. Within 34 to 20 days before parturition, up to 7% of all samples from each cow resulted in no coliform
isolated. Up to 11% of all samples from each cow resulted in 50 cfu/gm feces (i.e. one colony per plate) or less. No *E. coli* O157:H7 isolates were detectable throughout the study. Figure 1 shows levels of fecal coliform bacteria (log$_{10}$ cfu/gm feces) in each cow relative to calving date. Due to the variability in shedding in each animal, individual graphs were needed to clearly show the fluctuations in fecal coliforms (Figure 1).

**Study 2: Effects of feed intake on bacterial shedding in 7 periparturient cows** - A similar pattern for shedding of fecal coliforms was seen in these seven cows as was seen in the first study. Similar to the first study, up to 8% of all samples resulted in no coliform isolated from 5 of the 7 cows. Of these negative coliform samples, 90% occurred with samples obtained 34 to 15 days prior to parturition. The remaining percentage of negative samples occurred postpartum. Up to 24% of all samples from each cow resulted in 50 cfu/gm feces or less. (Figures 2a & 2b). Within 7 days of parturition, 5 of the 7 cows experienced a marked increase in fecal coliform shedding, with the other 2 cows showing peaks within 12 days of calving (Figures 2a & 2b). Paradoxically, no correlation was seen between fluctuations in feed intake and coliform shedding (overall correlation coefficient = -0.019; *P* = .79). Moreover, feed intake was not consistently altered in the cows (Figures 2a & 2b). Respective correlation coefficients for each animal in study 2 are shown in Table 1. Some cows exhibited decreased feed intake before the detected peak of coliform shedding, others after, and one cow never exhibited a decrease in her feed intake. No *E. coli* O157:H7 isolates were detected throughout the study.

**Discussion**

All 12 periparturient cows experienced large fluctuations in fecal coliform shedding with a $10^4$-$10^7$ increase in coliform numbers/gm of feces within 12 days of calving, coincident with the time when coliform IMI rates are reported to be the highest. In 7 of the 12 cows, coliform bacteria were completely undetectable in 4 - 8% of the fecal samples taken 34 to 15
days prior to calving. We believe this to be a conservative estimate of the frequency of samples negative for coliform bacteria because the sampling frequency was only 3-4 days per week. In agreement with earlier findings,\textsuperscript{25-28} it would appear that coliforms (in particular \textit{E. coli}) are not regularly present in high numbers in the feces of adult cows and are undetectable 4 - 8\% of the time. Current dogma would suggest \textit{coliform} bacteria would always be detectable in bovine feces. Our results, however, show fluctuations in fecal coliform shedding during the periparturient period.

Whether increased bacterial shedding is related to the well documented periparturient immunosuppression is not clear, however, the fact that both are occurring concurrently may explain higher IMI rates at this time. It has been established that IMI rates directly correlate with the number of bacteria in bedding\textsuperscript{20} and clinical infection from teat end exposure can result from only a minimal number of bacteria. Bovine mastitis can be efficiently achieved by experimentally infusing as few as 30 cfu of \textit{E. coli}\textsuperscript{29} With such minimal numbers of coliforms able to cause mastitis, the implications of results reported here on the relationship between exposure of the mammary gland to coliform bacteria and the high rate of IMI seen near parturition are noteworthy.

There is evidence that animals deprived of feed may exhibit increased coliform shedding.\textsuperscript{30,31} Brownlee and Grau in 1967 demonstrated that the incidence and magnitude of \textit{E. coli} and \textit{Salmonella} in the feces of cattle and sheep dramatically increased with feed intake restriction. Normally, the rumen represents a hostile environment for enteric bacteria, such as \textit{E. coli}, with only $10^3$-$10^6$ viable \textit{E. coli} cells/ml existing among the $10^9$-$10^{10}$ cell/ml of total bacteria. However, during periods of feed restriction, pH levels can exceed 7.0 and volatile fatty acids concentrations decline ($<$50 mM),\textsuperscript{32,33} both of which allow for \textit{E. coli} survival and growth. It has been reported that most periparturient cows exhibit a 15-30\% decline in dry matter feed intake around parturition,\textsuperscript{34} therefore we wondered if increased fecal coliform shedding was associated with a decline in feed intake. However, in study 2, the degree of
coliform shedding was not correlated with feed intake. (Table 1.) Further possible explanations for the transient peaks in fecal coliform shedding are limited. It seems unlikely that one factor alone can adequately explain the fluctuations in fecal shedding due to the numerous physiologic and immunologic changes occurring near parturition. These fluctuations may simply reflect fluctuations in substrate availability and utilization by the variety of microbial flora present in the ruminant gastrointestinal tract.

From a food safety viewpoint, the reliability of detecting *E. coli* as a monitor for fecal contamination may be questionable. Recently, the USDA Food Safety and Inspection Service (FSIS) has required licensed slaughter plants to test carcasses for *E. coli* as an indicator of the plants’ process control for fecal contamination. Fecal contamination is the primary avenue of carcass contamination by pathogenic microbes. *Escherichia coli* was chosen as a more appropriate organism to use as a verification of food safety than the originally proposed *Salmonella*-based testing. The FSIS concluded that since *E. coli* is present in all animal feces it is more effective than *Salmonella* as an indicator of fecal contamination. *Escherichia coli* is present in all mammals, however, our results suggest that this indicator for fecal contamination may be less than 100% accurate. Numerous time points in our study yielded negligible colony counts. Lack of *E. coli* detection for fecal contamination may therefore, represent a false negative for fecal contamination.

We had questioned whether shedding of coliform bacteria increased in the feces of periparturient cows. Increases in shedding of coliforms has been reported for fasting stress in cattle. An increased number of coliform bacteria in the environment of the cow and calf would represent a potential risk of new infection for both. Since *E. coli* O157:H7 is being reported as a normal intestinal resident of cattle, increased shedding of this isolate from periparturient cows might also represent a significant source of infection of the intestinal tract of the newborn calf. However, at no time in the current study was *E. coli* O157:H7 detected from these animals despite significantly elevated levels in total fecal coliform numbers. A recent study
estimated the herd prevalence of *E. coli* O157:H7 in cattle as being 0.3% to 0.7% and that the prevalence of animals within those herds ranges from 1.8% to 16%. Because the current study sampled only twelve cows, and the prevalence is very low in adult cattle, we believe this finding to be a logical outcome.

In summary, periparturient cows in our study experienced low, and at times undetectable, levels of fecal coliform shedding with a pronounced increase in coliform numbers near parturition. Our data demonstrate that coliform bacteria in feces are not uniformly shed, nor do all cows shed at similar levels. Regardless of level of shedding, all 12 cows experienced a $10^5$-$10^7$ cfu/gm of feces increase in fecal coliform bacteria shedding within 12 days of parturition. The increase does not seem to be related to feed intake. The increased shedding of fecal coliform bacteria seen near parturition may contribute to the high rate of new IMI of periparturient cows.

**References**


Table 1. Pearson product-moment correlations between feed intake and shedding of coliform bacteria in feces of periparturient cows. Data were recorded from 7 periparturient dairy cows beginning approximately 4-6 weeks prepartum and continuing through 2 weeks postpartum. Variations in fecal bacterial coliform shedding are not consistently correlated with changes in feed intake. Only 2 cows had a significant inverse relationship between fecal bacterial coliform shedding and changes in feed intake (*). Overall, there was no significant relationship between fecal bacterial coliform shedding and changes in feed intake.

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<td>Overall</td>
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Figure 1-- Fecal coliform shedding in periparturient cows: Study 1. Graphs represent levels of coliform bacteria colony-forming units (cfu) per gram of feces detected in relation to days relative to parturition. Note, Y-axes are different due to the variability of each cow.
Figure 2-- Fecal coliform shedding and feed intake in periparturient cows: Study 2. Graphs represent levels of coliform bacteria colony-forming units (cfu) per gram of feces detected in relation to days relative to parturition. Note. Y-axes are different due to the variability of each cow. The YY-axes represent amount of feed intake out of a total 18.18 kg of feed given to each cow daily.
Figure 2. (continued)
EXPRESSION OF NEUTROPHIL AND MONONUCLEAR CELL ADHESION MOLECULES IN CALVES TREATED WITH SELECTED NON STEROIDAL ANTI-INFLAMMATORY DRUGS AND DEXAMETHASONE

A paper to be submitted to the American Journal of Veterinary Research
L.S. Pelan-Mattocks and M.E. Kehrli, Jr.

Structured Abstract

Objective- To assess the effects of a single administration of selected non steroidal anti-inflammatory drugs (NSAIDS) versus a single dexamethasone administration on expression of bovine neutrophil and mononuclear cell adhesion molecules in vivo, and to assess various aspects of neutrophil function.

Animals- 22 healthy, Holstein calves.

Procedure- 15 calves were used per experimental trial and were randomly assigned to 3 treatment groups: control, dexamethasone, and NSAID. The experiment was repeated 4 times, each trial testing a different NSAID. Calves were administered a single dose of the

\[^a\] FACS® Lysis Solution. Becton Dickinson Immunocytometry Systems, Mountain View, Calif.

\[^b\] FACScan®. Becton Dickinson Immunocytometry Systems, Mountain View, Calif.

\[^c\] CellQuest®. Becton Dickinson Immunocytometry Systems, Mountain View, Calif.

\[^d\] Costar, Cambridge, Mass.

\[^e\] Quick-Count, Beragen.

\[^f\] Southern Biotechnology Associates, Birmingham, Alab.
respective treatments and expression of neutrophil and mononuclear adhesion molecules was assessed using flow cytometry. Additional measures of neutrophil function assessed were iodination, bacterial ingestion, and random and directed migration.

**Results**- A single injection of dexamethasone caused a neutrophilia due to shedding of the L-selectin (CD62L) adhesion molecule. Down-regulation of the $\beta_2$-integrin, CD18, was also observed. Conversely, NSAIDs had minimal effects on blood leukograms. Flunixin meglumine significantly increased CD18 expression. Aspirin, ibuprofen, and phenylbutazone increased CD62L expression on neutrophils 1, 2, and 3 days after treatment, respectively. Dexamethasone decreased the percentage of circulating $\gamma\delta$ T cells, whereas, aspirin and flunixin meglumine increased the percentage of circulating $\gamma\delta$ T cells.

**Conclusions**- The anti-inflammatory properties of NSAIDs tested in this study do not reduce neutrophil adhesion molecule expression nor seem to inhibit other aspects of neutrophil function that were tested. None of the NSAIDs altered neutrophil iodination, ingestion of bacteria or migration under agarose tests.

**Introduction**

A national survey conducted in 1992, showed that 93% of responding veterinarians used non steroidal anti-inflammatory drugs (NSAIDs) in treating food-producing animals, and over half reported prescribing NSAIDs more than once a week.\(^1\) Flunixin meglumine, dipyrone, aspirin, and phenylbutazone were the most frequently used NSAIDs.\(^1\) NSAIDs are commonly used for treatment of various disorders of cattle including diarrhea, septicemia, pneumonia, acute mastitis, lameness and other conditions accompanied by endotoxemia or pain.\(^2-5\) Inhibition of cyclooxygenase, the enzyme responsible for the biosynthesis of prostaglandins and related autocoids, is generally accepted to be the major mechanism of NSAID function.\(^6\) However, this mechanism does not fully explain all effects of NSAIDs.\(^7,8\)
Some NSAIDs have been reported to also interfere with various aspects of neutrophil function, including adhesion interactions.9-12 Recent studies have demonstrated that another category of anti-inflammatory agents, glucocorticoids, exert part of their anti-inflammatory affects on specific leukocyte adhesion molecules. This prevents neutrophil egress into tissues, thus blocking inflammation. The first committed step in inflammation is the adhesion of the leukocyte to the vascular endothelial wall followed by diapedesis of leukocytes into the extravascular space. Cellular adhesion molecules on both the endothelial wall and the leukocyte contribute to neutrophil egress into infected tissue. One adhesion molecule, L-selectin (CD62L) is present on bovine leukocytes and mediates neutrophil rolling along postcapillary venules under the shear force of blood flow thus forming the marginating pool of neutrophils.13 During an inflammatory response, neutrophil recruitment begins with the shedding of L-selectin and increased endothelial expression of the adhesion molecules E-selectin and P-selectin which bind weakly to neutrophil ligands.14,15 This weak interaction and exposure to inflammatory mediators increases the expression and adhesiveness of the β2-integrins (CD11/CD18) on neutrophils16 and triggers shedding of L-selectin. The β2-integrins (also known as leukocyte integrins), are a family of heterodimers composed of a CD11 α-chain and a CD18 β-chain. The α-chain can be either CD11a, CD11b, CD11c, or CD11d. The most abundant of the β2-integrins on neutrophils is CD11b/CD18 which mediates hyperadherence,17 while CD11a/CD18 is responsible for egress.18 Neutrophil integrins bind tightly to endothelial intercellular adhesion molecule-1 (ICAM-1) and migrate into the tissue toward a chemoattractant gradient. The importance of these molecular interactions for host defense is exemplified by individuals of various species lacking CD18 or L-selectin.19-23 Neutrophil recruitment in such individuals is profoundly impaired, predisposing them to a higher susceptibility to infection.

Glucocorticoids are also known to have immunosuppressive effects on leukocytes.24 Prolonged administration of glucocorticoids is contraindicated in animals with underlying
chronic infections due to the immunosuppressive effects on leukocytes which can cause subclinical infections to become acute clinical disease.\textsuperscript{25,26} If NSAIDs were also to have negative effects on leukocyte functions, their extended use in diseased animals could be contraindicated. Interference with adhesion molecule interactions in vitro has been reported for some NSAIDs.\textsuperscript{27-30} The purpose of this study was to assess and compare the effects of a single pharmacological dose of selected NSAIDs on leukocyte adhesion molecule expression and neutrophil functions with that of a single pharmacological dose of the synthetic glucocorticoid, dexamethasone.

**Materials and Methods**

*Calfes, treatments, and blood sample collections*

Twenty-two calves were used for this study in which there were: 4 trials, 3 treatments per trial and 5 calves per treatment. During each trial, only 15 of the 22 calves were randomly assigned to one of three treatment groups: control (no treatment), dexamethasone (0.04 mg/kg IM), or NSAID. A different NSAID, administered at standard pharmacological dosage, was tested for each trial: trial 1 = flunixin meglumine (1 mg/kg, intramuscularly), trial 2 = ibuprofen (25 mg/kg, intramuscularly), trial 3 = phenylbutazone (15 mg/kg, intravenously), and trial 4 = aspirin (31.2 mg, per os). After at least a two-week rest between trials, calves were either reassigned to a different experimental group or were replaced by a calf not tested in the previous trial. Therefore, no calf received an NSAID or dexamethasone treatment during two consecutive trials.

The trials lasted for 10 days in which a single dose of either dexamethasone or an NSAID was administered on the 5th day (day 0). Blood samples were collected by jugular venipuncture into 5 ml evacuated tubes containing acid-citrate-dextrose (ACD), at approximately 7 AM, on days prior to treatment (days -5, -4), immediately before treatment (day 0) and four consecutive days after treatment (days 1, 2, 3, 4).
Flow cytometric analysis

Round-bottom, 96-well plates were preloaded with 10 µl of monoclonal antibody (MoAb) (Table 1), one MoAb per well. Anticoagulated blood (50 µl) was added to each well and incubated at 39°C for 5 minutes. Erythrocytes were lysed by hypotonic lysis (200 µl of sterile distilled water for 30 seconds) and isotonicity restored with 20 µl of 10x saline. Plates were centrifuged (400 X g for 2 minutes) and supernatant was discarded. Next, 10 µl of the respective secondary FITC (fluoroisothiocyanate)-conjugated antibody was added and plates were incubated for 15 minutes in the dark at ambient temperature. After incubation, 100 µl of PBS was added to each well, and the plates were centrifuged and supernatant discarded. Cells were resuspended with 200 µl of Lysing Solution and plates were incubated for 15 minutes in the dark at room temperature. After incubation, contents of wells were transferred to 5 ml open-top round bottom tubes (one well/tube) each containing 100 µl of Lysing Solution and immediately analyzed by flow cytometry.

A flow cytometer was used to acquire data from 10,000 events per sample, and analysis was done using flow cytometric analysis software. Neutrophils and mononuclear populations were initially gated based on their forward and side scatter characteristics (FSC/SSC) (location indicative of the cell's size and internal complexity respectively) on dot plots. Erythrocytic and other cellular debris were gated out at this point. From mononuclear cell or granulocyte gates, subpopulations stained by the respective MoAbs were identified based on the FITC fluorescence histograms. Mean fluorescence intensities (MFI) of these positive staining cells were computed in the geometric linear mode. Histograms were plotted for each calf that represented background, CD62L, CD18, CD11a, CD11b, CD11c, and γδ T-cells. Adhesion molecule expression was assessed on each leukocyte gate and mononuclear cells were assessed for the percentage of γδ T-cells. Background fluorescence was identified and events to the right of this marker were considered positively stained cells.
Percentages of positive-staining cells and mean fluorescence intensity (MFI) data were recorded.

**Blood leukocyte and leukogram counts**

Total leukocyte counts (number of cells per microliter) in anticoagulated blood samples were determined by electronic counting. Differential leukocyte counts were determined by a whole blood assay assessed by flow cytometric analysis of CD45 (leukocyte common antigen) MFI and SSC.

Briefly, round-bottom, 96-well plates were preloaded with: 10 µl of murine anti-bovine CD45 (Table 1) and goat anti-mouse IgG2a-phycoerythrin. Anticoagulated blood from each animal (50 µl) was added to a well and plates were incubated in the dark for 5 minutes at 39°C. Following incubation, erythrocytes were lysed using 200 µl/well of cold pH-buffered H₂O for 30 seconds, and isotonicity was restored with 20 µl of 10X saline. Plates were then centrifuged (400 x g for 2 minutes) and supernatant discarded. Cells were resuspended in 100 µl of lysing solution and incubated at ambient temperature in the dark for 10 minutes. After incubation, content of wells were transferred to 5 ml open-top round bottom tubes (one well/tube) each containing 100 µl of lysing solution and immediately analyzed on a flow cytometer.

**Neutrophil function assays**

In vitro evaluations of neutrophil ingestion of ¹²⁵I-labeled, immunoglobulin-opsonized *Staphylococcus aureus* were performed as described. Assessments of opsonized zymosan phagocytosis-associated myeloperoxidase (MPO) catalyzed iodination. Random and directed neutrophil migration under agarose were assessed as described.
Analysis of data

Flow cytometry data was analyzed using Statistical Analysis System®, SAS Institute, Inc. The program PROC TABULATE was used to determine and summarize parameters including, means, frequency, and standard error of the mean. Data from each of the treatment groups were converted to a percentage of the control group mean for each test day to standardize for day-to-day laboratory variability. PROC ANOVA was used to perform analysis of variance. For MFI and percentage of gated cells, treatment effects were considered statistically significant at $P \leq 0.05$.

Results

Effects of dexamethasone and NSAIDs on leukograms

A leukocytosis was observed one day following dexamethasone injection (day 0) which was characterized as a neutrophilia (Figures 1a & 1b). Neutrophil cell numbers roughly tripled those of the control values. With flunixin meglumine being the only exception, the NSAIDs had no significant effect on leukograms (Figure 1a). Flunixin meglumine administration caused a significant 30% decrease in all leukocyte types.

Expression of neutrophil and mononuclear cell adhesion molecules and γδ T-cell receptor molecules

Dexamethasone treatment had the largest effects on neutrophil adhesion molecule expression, specifically in decreases in CD62L MFI, CD18 MFI, and in the percentage of γδ T-cells. Dexamethasone treatment throughout all trials roughly tripled the percentage of neutrophils. Throughout the study there was no effect of dexamethasone on the percentage of gated cells expressing any of the adhesion molecules; however, dexamethasone caused a significant decrease in the CD62L MFI (48%, 93%, 89%, 58% reductions for the 4 respective
trials) and a modest decrease in CD18 MFI (8%, 21%, 28%, 31% reductions for the 4 respective trials) (Figure 2a & b). In accordance with the decreased CD18 MFI expression, decreases in mean MFI expression of CD11a, CD11b, and CD11c were evident in all trials as a result of dexamethasone treatment. However, as a percent of the controls, only CD11a was statistically significant in trials 2 & 3 with reductions of 32% and 26%. There was a significant decrease in CD11b expression on neutrophils (40% decrease) only in trial 4.

Each NSAID treatment had unique effects on neutrophil adhesion molecule expression (Figure 2). Flunixin meglumine treatment increased expression of CD18 on neutrophils (105%). The effects of ibuprofen and phenylbutazone were minimal. Ibuprofen caused a 49% increase in CD62L expression on neutrophils on day 2 after treatment. Aspirin treatment caused an increase of CD62L MFI (38%) on neutrophils.

Expression of γδ T-cell receptor molecules.

Dexamethasone caused a marked reduction in the percent of γδ T-cells compared to controls in all trials. Changes in percent of control from day 0 to day 1 were significant in periods 2, 3, and 4, showing reductions of 73%, 57%, 53% respectively (Figure 2c). By comparison, ibuprofen also caused a decrease in number of positive gated mononuclear cells (79%), while also decreasing the MFI of these cells by 36%. Aspirin, however, caused the opposite response, and increased the proportion of γδ T-cells by 23%. Similarly, flunixin meglumine caused a 22% increase in the proportion of γδ T-cells.

Effects of dexamethasone and NSAIDs on selected neutrophil functions

Tests were not conclusive in assessing any variation caused by the single administration of dexamethasone nor with any of the NSAID treatments. There was no statistically significant difference found between any of the treatment groups (data not shown).
Discussion

Previous studies have assessed the effects of multiple injections of dexamethasone on expression of CD62L and CD18 on neutrophils. Dexamethasone treatment causes loss of stickiness to the endothelium and increases random migration of bovine neutrophils in vitro.\textsuperscript{24,32,33} Glucocorticoids, such as dexamethasone, are effective anti-inflammatory agents not only due to their down regulation of these neutrophil adhesion molecules but also because of their suppressive effects on neutrophil function. Glucocorticoids inhibit a phospholipase A\textsubscript{2} enzyme which inhibits release of arachadonic acid from cell membrane phospholipids and consequently inhibits arachidonic acid metabolism. It is well documented that in vivo glucocorticoid treatment results in the inhibition of ex vivo neutrophil function.\textsuperscript{32,34,35} Dexamethasone and cortisol both induce a neutrophilia in cattle without an increase in immature neutrophils,\textsuperscript{24} suggesting that glucocorticoids decrease the marginating pool of neutrophils, thus reducing the efficiency of egress of neutrophils from blood into tissues. In the present study, a single injection was potent enough to cause similar effects by down regulating expression of CD62L and CD18 on neutrophils. It was evident within one day after dexamethasone treatment that there was a simultaneous decrease in percentage of CD62L\textsuperscript{+} neutrophils and a tripling of the number of circulating neutrophils. Bovine studies, including the present one, indicate that a significant anti-inflammatory activity of dexamethasone is to decrease the size of the marginating pool of neutrophils by causing shedding of CD62L. The rapid onset and recovery of neutrophil CD62L expression also suggested that the down-regulating effect of a single dose of dexamethasone on CD62L is transient. Other mechanisms of action have been reported to explain some glucocorticoid anti-inflammatory properties. Anti-inflammatory effects of glucocorticoids may also be mediated in part by a monokine(s)\textsuperscript{33} and other mechanisms independent of arachidonic acid metabolism.\textsuperscript{36}

In agreement with previous studies, dexamethasone decreased the percentage of circulating γδ T cells. It has been reported that glucocorticoids cause a decline in the percentage
of circulating γδ T cells and a decrease in CD62L. MHC Class I & II molecules on remaining mononuclear cells. Since γδ T cells express high levels of CD62L and dexamethasone does not alter CD62L expression on γδ T cells, the egress of circulating γδ T cells into tissues during stress may represent a compensatory immunologic response when neutrophil egress is impaired.

The NSAIDs are a heterogeneous group of compounds, often chemically unrelated (although most of them are organic acids), which nevertheless share certain therapeutic actions and side effects. Inhibition of cyclooxygenase, the enzyme responsible for the biosynthesis of the prostaglandins and certain related autacoids, is generally thought to be the major mechanism of action of NSAIDs. This is in contrast to the glucocorticoids which inhibit an enzyme earlier in the arachidonic acid catabolism pathway. Each NSAID may, however, have additional mechanisms of action, and the sum of these different effects determines anti-inflammatory potency. Our data are consistent with the hypothesis that NSAIDs have differing mechanisms of action with respect to one another beyond their cyclooxygenase inhibition capability. In this study, the effects of NSAIDs as a group, contrasted with those of dexamethasone. NSAIDs had minimal effects on blood leukograms, with flunixin meglumine decreasing total leukocytes by roughly 30% from the control values. In contrast to dexamethasone treatment, flunixin meglumine increased CD18 expression on neutrophils, and aspirin, ibuprofen, and phenylbutazone increased CD62L MFI on neutrophils.

It was of interest that administration of NSAIDs markedly contrasted with the anti-inflammatory effects of dexamethasone. Since 1971, it has been believed that the major mechanism of action of NSAIDs is to inhibit the activity of cyclooxygenase (COX) within the prostaglandin production pathways. Cyclooxygenase has been found to be present in two isoforms. COX-1 is expressed constitutively, while COX-2 is induced in cells exposed to proinflammatory agents, including cytokines, mitogens, and endotoxin. Thus, the ability of NSAIDs in monogastric animals to inhibit COX-2 may explain their beneficial anti-
inflammatory effects. whereas inhibition of COX-1 may explain their unwanted side effect of gastrointestinal damage. Animals in the present study were apparently healthy with no underlying illness. Therefore, in absence of any induction of COX-2, the NSAIDs, were primarily inhibiting COX-1, which has not been shown to cause any negative effects on neutrophils. As shown in this study, increases in neutrophil adhesion molecule expression were apparent in the NSAID-treated animals that were not found in dexamethasone-treated nor control animals. Animals in this study were given only a single pharmacologic dose of the respective agents. It has been documented that low doses of aspirin and other NSAIDs markedly inhibit the synthesis of prostaglandins in vitro and in vivo whereas higher doses are required for an anti-inflammatory effect in vivo, suggesting that at higher concentrations NSAIDs exert an anti-inflammatory effect independent of cyclooxygenase inhibition. It has been suggested that the common pathway for anti-inflammatory drugs is the inhibition of neutrophil-endothelial and neutrophil-connective tissue interactions. Our results suggest, that with a single pharmacologic dose, some NSAIDs upregulating neutrophil adhesion molecule expression. Additionally, the finding that interfering with interactions involved in the adhesion of neutrophils to the microvasculature prevents NSAID-induced mucosal damage (in humans) has led to the proposal that such adhesion events occur early in the pathogenesis of NSAID-induced gastropathy. We speculate that the increase in expression of adhesion molecules, CD62L (induced by aspirin, ibuprofen, and phenylbutazone) and CD18 (induced by flunixin meglumine) may support to this hypothesis by priming or activating neutrophils for more rapid egress into tissues. It is also possible that the NSAIDs may aid neutrophil egress into inflammatory sites.

Results from murine and human in vitro research assessing NSAID mechanisms of action are not consistent. Diaz-Gonzalez et al suggest that NSAIDs' function is dependent upon shedding of L-selectin while Andrews et al suggest that NSAID mechanism is dependent upon their ability to upregulate ICAM-1. Fiorucci et al report NSAIDs upregulate
\( \beta_2 \)-integrin expression on human neutrophils, while Cronstein et al state that all NSAIDs inhibit neutrophil aggregation without affecting \( \beta_2 \)-integrins. \(^{29,42}\) Despite these conflicting in vitro studies, we believe our results are consistent with in vivo findings on treatment of inflammatory conditions in cattle (i.e., the mechanism of NSAIDs anti-inflammatory relief unrelated to impaired neutrophil adhesion molecule expression). \(^{3,5,45,46}\) Steroidal anti-inflammatory agents, such as glucocorticoids have been recommended in certain cases of acute mastitis \(^{17}\) but these have been shown to exert adverse immunosuppressive effects on leukocyte function. \(^{48}\)

Glucocorticoids, when administered to humans or animals, have been observed to decrease inflammation and to predispose to bacterial infection. \(^{32,49}\) There are many reports of neutrophil dysfunction after in vitro or in vivo exposure to glucocorticoids. Wesley et al reported that administration of dexamethasone to cows experimentally infected with \textit{Listeria monocytogenes} in the mammary gland actually increases milk somatic cell counts and increases bacterial shedding from mammary secretions. \(^{26}\) In contrast, other studies suggest that selected NSAIDs, not currently approved for intramammary use, are beneficial to bovine neutrophil function in vitro and may be advantageous in limiting mild production losses during mammary inflammation. Anderson et al have shown that multiple doses of flunixin meglumine improve clinical manifestations (by reducing rectal temperature, depression, and clinical signs of quarter inflammation) of acute mastitis induced by \textit{Escherichia coli} endotoxin and that adverse effects attributed to flunixin meglumine were not observed. \(^{4}\) Similarly, it has been shown that ibuprofen prevents endotoxin-induced pyrexia and provides some beneficial treatment in reducing heart and respiratory rates in similar cases. \(^{45}\) Importantly, impairment of neutrophil phagocytosis or function was not found in cows given flunixin meglumine or ibuprofen. \(^{4,45}\)

The NSAIDs we tested appear to allow for normal neutrophil adhesion molecule expression. Data suggest that immunosuppression accompanying environmental stress, pregnancy, calving, and administration of glucocorticoids like dexamethasone, can exacerbate
chronic bacterial infections and augment the risk of transmission of an infectious agent. While the precise anti-inflammatory mechanism of action in cattle remains to be elucidated for NSAIDs, our data suggest that aspirin, ibuprofen, flunixin meglumine, and phenylbutazone do not negatively affect neutrophil adhesion molecule expression.

References


Table 1. Monoclonal antibodies used to assess leukograms, neutrophil adhesion molecule expression and γδ T cell proportions.

<table>
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<td>F10-150</td>
</tr>
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<td>CD45</td>
<td>IgG2a</td>
<td>25 µg/ml</td>
<td>CACTB51A</td>
</tr>
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</table>

* Antibodies from Veterinary Medical Research Diagnostics, Pullman, WA
Figure 1-- The effects of selected anti-inflammatory drugs (NSAIDs) and a glucocorticoid, dexamethasone, on blood leukograms. Dexamethasone caused a leukocytosis as a result of a neutrophilia. Flunixin meglumine was the only NSAID to have an effect in leukogram counts, evident by causing a modest decrease in total leukocytes.
Figure 2-- The effects of selected anti-inflammatory drugs (NSAIDs) and a glucocorticoid, dexamethasone, on L-selectin MFI, CD18 MFI, and total number of percent positive γδ T cells. Dexamethasone caused a pronounced decrease in CD62L, while aspirin, ibuprofen, and phenylbutazone caused modest increases on days 1, 2, and 3 respectively. Dexamethasone caused a decrease in CD18 expression, while flunixin meglumine had a pronounced increase. Dexamethasone also caused a decrease in total number of percent positive γδ T cells, while aspirin was the only NSAID to have an effect with a modest increase in percent positive cells.
L-selectin MFI

CD18 MFI

+$\gamma$$\delta$ T cells

Days relative to administration of test article

-6 -5 -4 -3 -2 -1 0 1 2 3 4

* statistical significance at P≤0.05
RAPID BOVINE BLOOD LEUKOGRAM DETERMINATION USING FLOW CYTOMETRIC ANALYSIS OF CD45

A paper to be submitted to the *American Journal of Veterinary Research*

L. S. Pelan-Mattocks, B. A. Pesch, and M. E. Kehrli, Jr.

Structured Abstract

**Objective**---To develop an efficient and reliable method to determine bovine blood leukograms that accurately differentiates bovine lymphocytes from monocytes.

**Animals**---30 healthy Holstein and Jersey cows and 1 Holstein calf with bovine leukocyte adhesion deficiency.

**Procedure**---A flow cytometric method was adapted to assess differential leukocyte counts on bovine blood, combining CD45 (leukocyte common antigen) binding properties with the 90° light scatter properties on a flow cytometer. Conventional two-color flow cytometric analysis was done using selected monoclonal antibodies to verify that the locations of the

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*a* CellTrack, Angel Engineering, Corp, Trumbull, CT

*b* StatStain, Volu-Sol Corp., Henderson, NV

*c* Costar, Cambridge, MA

*d* FACS® Lysis Solution, Becton Dickinson, Mountain View, CA

*e* FACScan® Becton Dickinson, Mountain View, CA

*f* CellQuest Becton Dickinson, Mountain View, CA

*g* Version 6.12, SAS Institute Inc., Cary, NC, USA
leukocyte subpopulations determined by CD45 and side scatter characteristics were accurate. Secondly, a group of animals were analyzed by both conventional blood slide using light microscopy and by flow cytometric analysis, and correlation coefficients were determined to assess reliability of this procedure as compared to the conventional slide method.

Results--- CD45 and the side scatter properties of bovine leukocytes provides a clear differentiation of bovine leukocytes, including monocytes and lymphocytes.

Conclusions--- Unlike conventional light microscopy methods where discernment between bovine lymphocytes and bovine monocytes is not accurate, this method accurately differentiates the two mononuclear cell subpopulations. This assay is simple, rapid and importantly, it accurately distinguishes monocytes and lymphocytes. Fast preparation and analysis times allow for efficient and reliable examination of a large number of samples, and the laborious task of reading numerous slides is eliminated.

Introduction

Quantification of differential leukocyte counts by conventional microscope examination of stained blood slides is tedious and subject to technical variability. Technical inconsistencies are minimized by having well-stained slides evaluated by experienced individuals, and by performing differential counts on a minimum of 200 cells. Automated methods have been developed to increase the speed and accuracy of total and differential leukocyte counts in human blood, and more techniques are being modified to suit veterinary diagnostics. Despite the overall improvements in performance of differentiating hematology analyzers, they have often produced disappointing results in monocyte counting. Furthermore, conventional and novel techniques developed for humans and other species have proven to be less successful in cattle. It has been reported that bovine monocytes cannot be differentiated on blood smears stained with Wright's stain and are not distinguished from bovine lymphocytes by acid α-napthyl acetate esterase staining. Various methods have been
used to quantify the average proportion of monocytes among bovine blood mononuclear cells, however results have varied (percentages ranging from 3-19%) depending on the method used.12-16

Leukocyte differentials have been analyzed by flow cytometry on the basis of differences in cell size, cellular granularity, and intensity of fluorescence after staining with acridine orange in humans and other species, however, these methods yield similar difficulties in clearly distinguishing bovine mononuclear cell subpopulations.17-21 More successfully, fluorescent beads have been used to enumerate bovine monocytes via phagocytic capacity, yet cell preparation is time consuming.22

Recently, CD45 ("the leukocyte common antigen") has become a helpful tool for determining leukograms in human diagnostics, for which methods are continually being improved.23-25 CD45, an abundant transmembrane protein tyrosine phosphatase, is found on all leukocytes thus providing the basis for this method. In this report, an established concept has been adapted for the bovine, with promising implications for use in other large animals. This assay is simple and rapid; and more importantly, it is more accurate than the conventional blood slides method using light microscopy due to its ability to distinguish monocytes and lymphocytes clearly. Fast preparation and analysis times allow for efficient and reliable examination of a large number of samples, and the laborious task of reading numerous slides is eliminated. The present study assesses the reliability and accuracy of a flow cytometric technique adapted to the bovine to determine leukocyte subpopulations.

**Materials and Methods**

*Animals*---30 healthy Holstein (n=17) and Jersey (n=13) cows and one Holstein calf with bovine leukocyte adhesion deficiency were used as cell donors. Blood was obtained by jugular venipuncture into 5 ml evacuated acid-citrate-dextrose (ACD) tubes.
Total blood leukocyte counts and slide preparation for leukocyte differentials---Total leukocyte counts (cells/µL) were determined by electronic counting. Blood slides for light microscopy were prepared by use of a cytocentrifuge, stained with a combination of Wright's and Giemsa stains, and ≥ 200 cells were differentiated into neutrophils, eosinophils, or mononuclear cells (this method has been reported to not accurately discern lymphocytes from monocytes in bovine blood). The relative proportions and total numbers of each cell population per microliter of blood were calculated. Slides were also counted in duplicate by two qualified individuals to assess human variability.

Blood leukogram determination---For CD45 staining process, 96-well round-bottom plates were preloaded with: 10 µl of murine anti-bovine CD45 and 10 µl of goat anti-mouse IgG2a (Table 1). Anticoagulated blood from each animal (50 µl) was added per well and plates were incubated in the dark for 5 minutes at 39°C. Following incubation, erythrocytes were lysed using 200 µl/well of cold H2O for 30 seconds, and isotonicity was restored with 20 µl of 10X saline. Plates were then centrifuged (400 x g for 2 minutes) and supernatant was discarded. Cells were resuspended in 200 µl of Lysing Solution and incubated in the dark at room temperature for 10 minutes. After incubation, wells were transferred to 5 ml test tubes (one well/tube). Cells were analyzed on flow cytometer immediately after resuspension and transfer.

A flow cytometer was used to acquire data from 5,000-10,000 events per sample, and software was used for fluorescence analyses. Leukocyte populations were initially gated based on their forward scatter (relative particle size) and side scatter (intracellular complexity) characteristics (FSC/SSC) on dot plots. Erythrocytic and other cellular debris were gated out at this point. From this, subpopulations were subsequently gated based on SSC and CD45 specific fluorescence.

Verification of subset population location---Conventional two-color flow cytometric analysis was done using the aforementioned protocol except with selected monoclonal
antibodies (Table 1) to verify the locations of the subset populations. Note that different from the CD45 differential process, plates were loaded with 75 µl of each primary antibody, and 100 µl of whole blood. Appropriate secondary antibodies (75 µl) were added after the supernatant was discarded from the first lysis step. Plates were then incubated in the dark at ambient temperature for 15 minutes. Plates were then centrifuged, and supernatant discarded. Cells were resuspended in Lysis solution and protocol continued as described.

Statistical analysis—Pearson product-moment correlation coefficients were used to assess variability between the two methods. Variability within a method and between the two slide readers were determined using PROC CORR of SAS.

Results

The range of leukocyte counts from the 31 animals evaluated was 5,200/µl to 68,700/µl. As determined by conventional light microscopy, the average percentage of neutrophils was 33%, 7% eosinophils and 59% mononuclear cells, which was significantly correlated with 39% neutrophils ($r=0.90$), 3% eosinophils ($r=0.80$), and 56% mononuclear cells ($r=0.82$) as determined by CD45 flow cytometry analysis.

Monocyte and lymphocyte percentages were not determined by the conventional light microscopy method, but by flow cytometry they represented averages of 47% lymphocytes and 10% monocytes. Two color flow cytometric analysis was used to confirm the percentages of the lymphocytes and monocytes and to identify the general location of B cells, T cells, CD4+ T cells, CD8+ T cells, and $\gamma$δ T cells. Based upon CD45 MFI and SSC (illustrated in Figure 1), monocytes fell into a distinct clustering between granulocytes and lymphocytes. Two color staining using a monoclonal antibody recognizing CD14 (a monocyte marker) confirms this location having 98% of the cells in this cluster being CD14+. CD3 and CD8, marking lymphocytes, are shown in red on the bottom of their respective graphs. CD4 seemingly marked two subpopulations in CD45 vs SSC. Although faintly staining, it illuminates
eosinophils. The N12 monoclonal antibody and the monoclonal versus B cells also appear to be binding eosinophils. Lastly, there is no discrepancy that the granulocyte marker clearly shows the granulocytes.

There were high correlations between the blood slides using light microscopy and the CD45 method for all leukocyte subclasses except for the mononuclear cell subpopulations. Correlation coefficients are listed in Table 1. Variability in individuals reading slides was seen in the correlation coefficients between the individual readers. \((r= .91)\), whereas when a sample was repeated and analyzed via the flow cytometer, population percentage differences were negligible \((r= .99)\).

**Discussion**

The CD45 staining technique reported here represents a significant improvement over conventional light microscopy differentials and is supported with the following: first, each major cell population, including monocytes, was found in a discrete region. By gating these regions, population percentages were readily determined by the software. Second, precision is enhanced by examining 5000 cells or more, rather than the 200-300 cells examined on the microscope. Third, the processing time is significantly shorter than slide preparation and light microscopic differentiation. The possibility for delaying analysis for a few hours after sample preparation is also an advantage. Once cells are fixed by the Lysis solution, analysis can be delayed for at least 6 hours. Additionally, plates can be prepared in advance and stored sealed at 4°C for at least 2 weeks. Major limitations of this method are that visual assessment of leukocyte morphology cannot be made and a flow cytometer is required. This technique represents a powerful and rapid technique for research laboratories. In assessing 5 animals, we spent approximately 2 hours completing conventional slide preparation and analysis whereas the same took 20 minutes using the CD45 flow cytometric technique.
As stated, one of the major advantages of the CD45 flow cytometric technique is the ability to accurately distinguish mononuclear populations. Monocyte and lymphocyte percentages could not accurately be determined by the conventional light microscopy method, but by flow cytometry they represented averages of 47% lymphocytes and 10% monocytes. Two color flow cytometric analysis was used to confirm the percentages of the lymphocytes and monocytes and to identify the general location of B cells, T cells, CD4+ T cells, CD8+ T cells, and γδ T cells. Based upon CD45 MFI and SSC and illustrated in Figure 1, monocytes fell into a distinct clustering between granulocytes and lymphocytes. Two color staining using a monoclonal antibody recognizing CD14 confirms this location. This cluster was 98% CD14+ indicating that 98% of the cells in this region were monocytes. CD3 and CD8, clearly marked lymphocytes. CD4 marked two subpopulations in CD45 vs SSC. Although faintly staining, it appears on a population which we believe to be eosinophils. This has been documented with human and swine eosinophils which normally express CD4.26-28 The N12 monoclonal antibody and the monoclonal versus B cells also appears to be binding eosinophils. Normally this upper region is gated out and ignored when analyzing CD4 staining of lymphocytes and this binding by a primarily mononuclear marker is often missed. The granulocyte marker clearly demarcates their subset.

There were high correlations between the blood slides using light microscopy and the CD45 method for all leukocyte subclasses except for the mononuclear cell subpopulations, as expected. Correlation coefficients are listed in Table 1. The conventional light microscopy method consistently yielded higher counts for eosinophils than the flow cytometric method, thereby resulting in the lowest correlation coefficient of all the cell populations assessed. This could be explained by the tendency for the human eye to be attracted to brightly staining eosinophils compared to other less brightly stained cells. This “error” is evident by the variability seen between readers.
The accuracy of differential leukocyte counts determined by flow cytometry has been increased using the monoclonal antibody against CD45. The current study clearly presents complete and accurate differential leukocyte counts from healthy animals and detects abnormalities in leukograms such as the neutrophilia exhibited by diseased animals. The method described herein allows for analysis of large numbers of blood specimens, and stores data in a computerized database. Most importantly, it is reproducible and greatly reduces labor requirements.

References


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*VMRD, Pullman, WA*
Table 2. Pearson product-moment correlations between conventional slide differentials and flow cytometric differentials were determined. Comparison of leukogram data between the two methods shows that both are highly correlated. Lymphocyte and monocyte values could not be included in the correlation coefficients due to the inability of the slide method to distinguish these subpopulations. Data were recorded from 31 animals.

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<td><strong>Overall</strong></td>
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Figure 1 -- Two-color flow cytometric analysis of bovine leukocytes stained with selected leukocyte subset specific, FITC-labeled monoclonal antibodies and phycoerythrin-conjugated anti-CD45 monoclonal antibody. The larger dot plot to the upper left shows various leukocyte populations separated into distinct groups by CD45 and side scatter characteristics. The seven smaller dot plots and histograms (using monoclonal antibodies for CD3, CD8, CD14, CD4, anti-gamma-deltal-1, anti-B cell, and granulocytes respectively) provide verification that each distinct subpopulation is correctly identified.
GENERAL SUMMARY

Stress in cattle is associated with increased susceptibility to infectious disease. Neutrophils are a vital to the inflammatory response, and impaired neutrophil function is associated with predisposition to new intramammary infection, respiratory disease, and other illness. Periparturient cows have a high incidence of coliform mastitis and newborn calves can experience diarrhea caused by coliform bacteria. Contrary to current beliefs, our data demonstrate considerable variability in shedding of coliform bacteria in feces in adult cows. All cows studied, experienced a $10^4$-$10^7$ cfu/gm of feces increase in fecal coliform bacteria shedding within 12 days of parturition, suggesting increased shedding of fecal coliform bacteria near parturition may contribute to the increased rate of coliform infections in periparturient cows and calves. Immunosuppression induced by anti-inflammatory drugs can exacerbate subclinical infections into clinical disease. NSAIDs are commonly used for treatment of various conditions accompanied by endotoxemia or pain. If NSAIDs have negative effects on leukocyte function, their extended use in diseased animals could be contraindicated. Our results indicate that aspirin, ibuprofen, flunixin meglumine, and phenylbutazone have no negative effects on neutrophil adhesion molecule expression or other neutrophil functions tested. In our final study, we evaluated an efficient and reliable method to determine bovine blood leukograms, that accurately differentiates bovine lymphocytes from monocytes. A flow cytometric method was adapted to assess differential leukocyte counts on bovine blood, using CD45 (leukocyte common antigen). Fast preparation and analysis times allow for efficient and reliable examination of a large number of samples, and the laborious task of reading numerous slides is eliminated.
APPENDIX: THE EFFECTS OF FASTING STRESS AND DEXAMETHASONE ON NEUTROPHIL CD62L EXPRESSION IN CALVES HARBORING E. COLI O157:H7

L.S. Pelan, M.E. Kehrl, Jr., T.A. Casey, B.T. Bosworth

Abstract

In simulating pre-slaughter livestock feeding schedules and stress, we studied the interaction between the ability to mount an inflammatory response and fecal coliform shedding in cattle harboring Escherichia coli O157:H7. Minor fasting has been shown to change rumen pH and volatile fatty acid levels thus optimizing conditions for E. coli O157:H7 growth. Glucocorticoids induce neutrophils to shed L-selectin (CD62L), thus reducing the efficiency of normal inflammatory responses. We monitored CD62L during each experiment to see if loss of CD62L on neutrophils was correlated with increased fecal coliform shedding. Twelve Holstein calves 4-6 months old, were inoculated with $10^7$-$10^{10}$ cfu E. coli O157:H7 and subjected to a fluctuating feeding schedule over 5 days after which they were returned to a normal feeding schedule. Eight of these calves were then given a single injection of dexamethasone (0.04 mg/kg, IM) after a 2 week rest. We found that dietary stress increased fecal coliform shedding (including E. coli O157:H7) and that neutrophil CD62L expression was only affected by dexamethasone administration. Within 24 hours, dexamethasone depressed the proportion of neutrophils bearing CD62L by approximately 75%. A stress leukogram was observed during both fasting and dexamethasone treatments. The basis of increased fecal coliform shedding appears to be different for the two stressors.
Figure 1 -- The effects of dexamethasone on fecal coliform shedding. Dexamethasone allows for fecal coliform bacteria levels to remain higher for a longer period of time than controls.
Colon bacteria/gm feces (Log_{10})

- Controls
- Dexamethasone

Days relative to treatment
Figure 2 -- The effects of dexamethasone on the mean fluorescent intensity (MFI) of CD62L and the effects of dexamethasone on the number of cells expressing CD62L.
Controls

Dexamethasone

CD62L MFI

%CD62L+

Days relative to treatment
ACKNOWLEDGMENTS

I would like to express my sincerest thanks and appreciation to Dr. Marcus Kehrli, Jr. for his encouragement, guidance, and undaunting humor. I am indebted to him for his support and trust. I would not have reached this point without him. He has become more than a mentor; he has become a respected friend. I would also like to thank Dr. James A. Roth for being a supportive and insightful co-major professor. I thank him for taking the time to talk to me as a Penn State undergraduate about Iowa State University during his visit to State College, PA. To the other members of my committee, Dr. Tom Casey, and Dr. Mark Rasmussen, thank you for providing valuable advice and encouragement. I would also like to thank Dr. Jesse P. Goff for his insight into my projects, for teaching me a great deal about veterinary medicine, and for always having time to talk. Thank you, I have truly learned so very much.

I feel very fortunate to have had the opportunity to work at the National Animal Disease Center, USDA-Agricultural Research Service. I was impressed not only with the number of talented scientists but also with their open willingness to offer advice and assistance. I owe a debt of gratitude to Bruce Pesch for teaching me some of his flow cytometric knowledge, and I would like to thank Norman S. Tjelmeland, Creig E. Caruth, Eugene C. Rieks, and Bruce Gray for helping me care for my research animals and for giving me more insight and experience in dairy husbandry. I would also like to extend a huge thanks to Arlen J. Anderson for his assistance in the lab and in the barns, and for acclimating me to Iowa, i.e. teaching me how to tell north, south, east, and west -without looking for the sun. In addition, my colleges, Eun-kyung Lee, Veena Rajaraman, Nancy Maroushek-Boury, Haa-Yung Lee, Kayoko Kimura, Mohammad Heidari, Anton Roach, Simon Mwangi, and In-kyung Lee have been a tremendous help toward my project and education. They have not only helped me keep my projects running smoothly, but also provided encouragement, laughter, and friendship.
I would like to thank Dr. David F. Cox, who while teaching me a bit about SAS and statistics, managed to teach (and remind) me more about the “important things in life”. His encouraging words have meant so very much. Maybe someday I will find some way of repayment. (No, the straw doesn’t count.)

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And last, I would like to offer a very special thanks to my husband, Ryan, for all his love and faith that helped me see this degree to its completion. He has all of my heart, now and always. I look forward to the rest of our lives...Amor vincit omnia.