

The Impact of Calcium and Vitamin D on the Immune System

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INTRODUCTION

The dairy industry is the second largest animal agriculture commodity produced in the United States and is equal to corn in economic impact. Therefore, issues that affect the health of the dairy cow are of great importance to the economy of the country. The dairy cow is most vulnerable to illness during transition, because it is during this period of time that the cow's immune system is functionally suppressed. Immunosuppression contributes to higher incidence of infectious and metabolic diseases; the most common disease in the transition dairy cow is mastitis. The economic impact of mastitis alone on the dairy industry is estimated to be 2 billion dollars/yr.

THE PERIPARTURIENT PERIOD

Factors such as pregnancy, parturition, blood Ca levels, initiation of lactation, and nutrition all affect the ability of the cow's immune system to effectively combat infections. We have systematically studied parts of the immune system and have explored the affects of Ca, lactation, parturition, and nutrition factors on the immune system. The periparturient period is the time where these complex physiological changes occur simultaneously, having a significant effect on the animal's health. Numerous studies have demonstrated that the immune system of a dairy cow around the time of calving is suppressed. Beginning about 1 to 2 wk before calving and lasting until between 2 and 4 wk after calving, numerous immune functions have been shown to be inhibited during this time period (Kehrli et al., 1989). Impaired immune cell functions during this periparturient period contribute to new infections leading to such diseases as mastitis and metritis (Oliver et al., 1988).

ASSOCIATION BETWEEN METABOLIC DISEASES AND IMMUNITY

Several epidemiological studies have shown an association between a diagnosed metabolic disease and subsequent development of mastitis. One metabolic disease that has been associated with immune system disorder is hypocalcemia or milk

fever. A study of over 2000 cows showed that cows with hypocalcemia were 8 times more likely to develop mastitis than cows with normal blood Ca levels (Curtis et al., 1985). Severe hypocalcemia leads to the loss of proper skeletal muscle control. Clinical hypocalcemia occurs in 5 - 7 % of transition dairy cows. Additionally, subclinical hypocalcemia occurs in 25 % of heifers and > 40 % of second lactation cows (Reinhardt et al., 2011). Contraction rate and strength of smooth muscle tissue have been shown to be directly related to the level of Ca in the blood (Daniel, 1983). A current hypothesis is that even a sub-clinical hypocalcemic cow would have decreased muscle tone in the smooth muscle that makes up the teat sphincter and that this loss of muscle tone would cause the teat canal to remain partially open; thus exposing the mammary gland to environmental pathogens.

In addition to Ca's critical role in muscle function, it also plays an essential role in intracellular signaling. In immune cells, intracellular Ca regulates many cellular functions including: cytokine production, cytokine receptor expression, and cell proliferation. Recently it has been shown that stimulated peripheral mononuclear cells from hypocalcemic cows have a muted intracellular Ca response compared to cows with normal blood Ca levels. Furthermore, when stimulated peripheral mononuclear cells from hypocalcemic cows were compared with stimulated peripheral mononuclear cells obtained from the same cows after intravenous treatment with a Ca solution, a muted intracellular Ca response was demonstrated only when the animals were hypocalcemic (Kimura et al., 2006). A muted intracellular Ca response would have a significant effect on the functional capacity of the cells of the immune system.

Maintenance of proper blood Ca levels is critical for an animal's health. There are 2 effective means of preventing periparturient hypocalcemia, both of which are diets used in the weeks prior to calving. The first diet reduces Ca intake prior to calving. The theory to this approach is that a mild hypocalcemia prior to lactation will cause the animal's Ca transport machineries to up-regulate, making the absorption of Ca more efficient (Green et al., 1981). Then as lactation begins and the demand for Ca rapidly

increases, the capacity for Ca transport has already been increased. The second means of preventing periparturient hypocalcemia is the use of the Dietary Cation-Anion Difference (DCAD) diet. Researchers in the early 1970's showed that the use of anionic salts in the diet could prevent hypocalcemia in dairy cows (Dishington, 1975). Since that time numerous studies have shown that adjustment of the cation-anion balance can reduce the incidence of hypocalcemia seen in periparturient cows (Block, 1984; Goff et al., 1991).

THE AFFECT OF LACTATION ON THE IMMUNE SYSTEM

To study the affect of lactation on the immune system, normal dairy cows were compared to cows that had undergone a mastectomy. Specific immune cell (lymphocytes or neutrophil) function was assessed in the mastectomized animals and compared to normal animals. Studies showed that lymphocyte function was significantly different in mastectomized animals compared to normal animals during transition. This demonstrated that the depression of lymphocyte function during the periparturient period could largely be attributed to the metabolic demands of milk production. These observations fit nicely with the molecular observations described above showing hypocalcemia's effect on lymphocyte intracellular Ca transport. In contrast to the lymphocytes, neutrophils showed a decrease in function starting about 2 wk prior to calving and reaching the low point at the time of calving in both mastectomized and normal cows. However, mastectomized cows quickly recovered neutrophil function (7 d); whereas, normal animals had not recovered neutrophil function after 20 d (Kimura et al., 1999). These data suggest that lactation plays a significant role in the recovery phase of periparturient immunosuppression. Importantly, the absence of the mammary gland did not affect the manifestation of periparturient immunosuppression, but only affected the duration of the suppression after calving. Therefore, the Ca imbalances that typically affect the immune system are not the only causes of suppression of neutrophil function.

THE AFFECT OF PREGNANCY ON IMMUNE CELL FUNCTIONS

Studies have shown that pregnancy alters immune cell functionality. In fact, immune suppression has been long thought to be necessary in maintenance of pregnancy and that break down of immune suppression is one factor that may be

involved in spontaneous abortions. Pregnancy is known to exacerbate some human autoimmune diseases (e.g. Systemic Lupus Erythematosus) and ameliorate other diseases (e.g. rheumatoid arthritis). The mechanisms for these alterations of immune cell function are unknown.

Polymorphonuclear neutrophils are a type of immune cell that is of great importance to the health of the dairy cow. The neutrophil is the first line of defense in those pathogens that cause the majority of diseases that affect production in the dairy cow. Research has shown that pregnancy affects the functional capacity of neutrophils. Stimulated neutrophils from pregnant women showed significantly less respiratory burst activity (a primary pathogen killing mechanism) compared to a control group (Crouch et al., 1995). Similarly, 2 enzymes in the hexose monophosphate shunt that is part of the pathway that produces NADPH required for respiratory burst activity, have been shown to be localized to different subcellular areas in pregnant versus non-pregnant women (Kindzelskii et al., 2004). Because pregnancy can affect important functions of immune cells and because of the reproduction schedule used with dairy cattle, future research into the effects of pregnancy in dairy cattle are critical to the understanding of the animal's immune system. Recently the intracellular location of myeloperoxidase, an enzyme critical to respiratory burst, was altered in pregnant women (Kindzelskii et al., 2006). It is unknown why this occurs and what affect it has on the pregnancy.

SUPPRESSION OF NEUTROPHIL FUNCTION

Periparturient immunosuppression is manifest in a wide range of immunological dysfunctions, including impaired neutrophil and lymphocyte functions (Kehrli and Goff, 1989; Mehrzad et al., 2001; and Shuster et al., 1996). As part of the innate immune system, the neutrophil is an essential first responder to infection and is considered vital to effective clearance of bacteria from the mammary gland (Paape et al., 2003; and Mollinedo et al., 1999). Neutrophils have various killing mechanisms to destroy pathogens (Segal, 2005). Upon encountering invading bacteria, neutrophils will ingest the bacteria into phagosomes that are fused with lysosomes. This process stimulates neutrophils to produce large amounts of oxidizing agents in a process referred to as *respiratory burst* in which oxygen radicals are generated that serve as precursors to various antimicrobial oxidants. In addition to

oxidizing agents, neutrophils contain numerous antimicrobial granule proteins such as cathelicidins, hydrolases, proteases, lactoferrin, and lysozyme. These proteins are either released into phagosomes to destroy ingested pathogens, or the granular contents are released out of the cell. These neutrophil functions are suppressed at and around the time of parturition (Kehrli and Goff, 1989; Mehrzad et al., 2001; and Shuster et al., 1996). Reduced neutrophil function and its association with periparturient immunosuppression is a significant problem for the transition dairy cow. This immunosuppression is associated with an increased susceptibility to mastitis.

FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS IN MILK

Although neutrophils play an important role in the control of an infection in the mammary gland, neutrophils isolated from milk show inhibited functionality compared to those isolated from blood (Paape et al., 2003). Neutrophils isolated from milk have a decreased ability to kill bacteria *in vitro* (Paape and Guidry, 1977) and have a decreased capacity to generate reactive oxygen species upon stimulation (Mehrzad et al., 2001). When blood neutrophils are incubated in milk *in vitro*, milk's inhibitory effects on neutrophil function are quickly manifest. Experiments with addition of casein to blood neutrophils showed that only a 5 min pre-incubation with casein resulted in reduced reactive oxygen species generation following stimulation (Cooray, 1996). Furthermore, neutrophils incubated in skim milk, whey, or media were more able to ingest bacteria than neutrophils incubated in whole milk (Paape and Guidry, 1977; and Paape et al., 1975.).

Neutrophil extracellular traps (NET) are a recently described mechanism by which neutrophils kill bacteria (Brinkmann et al., 2004). Neutrophils stimulated with PMA or IL-8 release nuclear and granular materials forming extracellular fibers that trap and kill bacteria. These NET form a web of DNA, histones, and other nuclear and granular proteins that have been shown to have antibacterial activity. To support this idea that NET have antibacterial activity, it was shown that neutrophil killing of bacteria was significantly reduced with the addition of DNase *in vitro* (Brinkmann et al., 2004). Further support for bactericidal action of extruded DNA was provided by a study demonstrating that a bacterial strain with deletions of the genes encoding multiple DNases that are secreted by the bacteria was much less virulent than the wild-type strain (Sumby

et al., 2005). Recently we have shown that bovine neutrophils generate NET following stimulation and more importantly that these NET can form when the neutrophils are incubated in whole milk. Our data shows that neutrophils pre-incubated for up to 6 hr in milk prior to stimulation retain their ability to cast NET (Lippolis et al., 2006). This is a significant contrast to the ability of casein to quickly (5 min) inhibit neutrophil oxidative burst (Cooray, 1996) or for milk to inhibit phagocytosis (Paape et al., 1975). With other bacterial killing mechanisms inhibited by milk, the neutrophil may rely more on the NET to kill bacteria invading the mammary gland than perhaps in other tissues.

THE ROLE OF VITAMIN D IN IMMUNE FUNCTION

The physiological role of the vitamin D system continues to evolve beyond Ca and skeletal homeostasis to include significant roles in modulating innate and adaptive immune function (Figure 1). Most significant is the finding in humans that the levels of 25(OH)D₃ required for optimal immune response are greater than those for Ca and skeletal homeostasis. It has long been recognized that vitamin D deficiency, as reflected in serum 25(OH)D₃ concentrations, causes decreased resistance to infection (Reinhardt and Hustmyer, 1987; and Rook et al., 1986), but this action was generally thought to be secondary to endocrine effects of vitamin D on Ca metabolism. The active form of vitamin D, 1,25-dihydroxy vitamin D₃ (**1,25(OH)₂D₃**), has also been used as an adjuvant, and it was shown that treatment of cows with the active form of vitamin D along with an *E. coli* vaccine strain resulted in greater levels of antibodies specific for the vaccine in milk and serum compared to vaccine alone (Reinhardt et al., 1999).

Recently, vitamin D has been shown to play a role in regulating gene expression in immune cells and their ability to kill pathogens. Serum levels of the major form of vitamin D, 25(OH)D₃, have been correlated with the efficacy of human macrophages to kill *Mycobacterium tuberculosis* in culture (Liu et al., 2006). Those patients with low 25(OH)D₃ had immune cells that did not effectively kill bacteria; whereas those patients with higher serum levels of 25(OH)D₃ were able to kill bacteria. It was shown that the addition of 25(OH)D₃ to serum deficient in 25(OH)D₃ was sufficient to restore the ability of macrophages to kill the bacteria in culture. Screening of human and mouse genomes revealed over 3,000 genes with a vitamin D response element to which

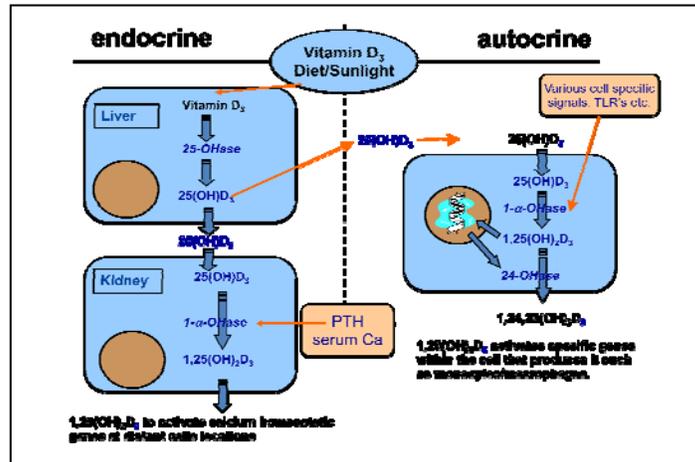


Figure 1: The endocrine and autocrine vitamin D pathways. The endocrine pathway affects Ca homeostasis; whereas, the autocrine pathway affects immune cell function.

1,25(OH)₂D₃ in combination with the vitamin D receptor (**VDR**) affect gene expression (Wang et al., 2005), some of which are involved in immune cell regulation. Currently, vitamin D status as measured by serum 25(OH)D₃ is thought to play a role in various disease states; such as various infectious diseases, cancers, and autoimmune diseases (Plum and Deluca, 2010).

Vitamin D has long been studied for its role in Ca homeostasis. It was thought that the only source of the enzyme 1-alpha hydroxylase (**1α-OHase**), the enzyme that catalyzes the conversion of 25(OH)D₃ to the biologically active steroid hormone 1, 25(OH)₂D₃ were kidney cells. The 1,25(OH)₂D₃ then binds to VDR, and this complex binds to a DNA sequence motif in the promoter regions of specific genes. However, recent data has shown that monocytes and macrophages can express 1α-OHase in response to activation by interferon or signaling through the toll-like receptor (**TLR**) pathways (Liu et al., 2006). Toll-like receptors are receptors found on most cell types that bind to various molecules associated with pathogens. For example, TLR-4 binds to lipopolysaccharide (**LPS**), a molecule found on gram-negative bacteria, and causes various immune related gene expression changes in both immune and non-immune cells. TLR signaling is thought to be one of the first signals to the host of an infection and thus starts the host immune response (Goldman, 2007; Trinchieri and Sher, 2007). What is known is that 25(OH)D₃ can enter a monocyte/macrophage and upon detection of a pathogen through the TLR pathway 1α-OHase is expressed, which converts the 25(OH)D₃ to 1,25(OH)₂D₃. The 1,25(OH)₂D₃ then

binds to VDR in the immune cell and the complex directly affects gene regulation by binding to specific regions of the genome. We have shown that adding LPS, or other TLR ligands, causes bovine monocytes to express 1α-OHase (Nelson et al., 2010). Furthermore, we have shown that the expression of specific genes is dependent on the concentration of 25(OH)D₃ in media. Importantly, we have shown via RT-PCR that there are differences in the types of genes expressed in bovine monocytes compared to human monocytes. For example, it has been shown that the cathelicidin gene expression increases in human monocytes and that this protein plays an important role in killing the intracellular *M. tuberculosis* bacterium (Liu et al., 2006). However, in bovine there are multiple cathelicidin genes (Zanetti, 2004). We were able to distinguish the expression of 3 of the bovine cathelicidin genes with RT-PCR analysis and showed the expression of these genes are not affected by TLR mediated 1,25(OH)₂D₃ production (Nelson et al., 2010). Research that would add to the knowledge base of genes whose expression is affected by 25(OH)D₃ concentration and subsequent TLR mediated 1,25(OH)₂D₃ production would be very helpful in the understanding of the role of vitamin D on the bovine immune system.

In addition to the *in vitro* work above, we have shown that tissue from an infected mammary gland and CD14+ cells (monocytes) obtained from milk from infected animals have up-regulated expression of 1α-OHase (Nelson et al., 2010). Since direct detection of intercellular levels of 1,25(OH)₂D₃ that are sufficient for gene regulation is not yet possible, indirect evidence, such as detection of a gene whose

expression is dependent on 1,25(OH)₂D₃ is the standard in the field. Therefore, demonstrating expression of a gene (**Cyp24A1**) that is dependent on the production of 1,25(OH)₂D₃ showed for the first time in any species that the vitamin D pathway is active *in vivo* during a bacterial infection (Nelson et al., 2010). Importantly in the presence of added exogenous 25(OH)D₃ these CD14+ cells produce 1,25(OH)₂D₃, which results in increased expression of the iNOS and Rantes genes, as well as possibly other genes that may influence the outcome of an infection, based on data from humans and bovine monocytes (Nelson et al., 2010). In mammary infections large numbers of immune cells can be obtained from the infection site via milk collection. In a typical uninfected mammary gland somatic cell counts can be from 20,000 to 50,000 cells/ml for an uninfected quarter, infected gland can have millions of cells/ml. Monocytes constitute approximately 30 - 40 % of the cell count in an uninfected gland and 8 - 10 % in an infected quarter. With the ability to obtain liters of milk, sufficient cell numbers can be isolated to do cell sorting and subsequent RNA analysis (Nelson et al., 2010). Additionally, samples from milk consist of immune cells that have been recruited to and activated at the site of the infection, as opposed to blood samples that consist of immune cells that may or may not be targeting the site of infection.

In all, we have shown vitamin D does affect bovine immune cells causing gene expression changes. Our hypothesis is that vitamin D status in the bovine can be an important indicator of immune functionality.

SUMMARY

The occurrence of a severe suppression of the immune system during the periparturient period in many dairy cows is well documented. This loss of immune function is strongly associated with the occurrence of diseases that take advantage of the weakened immune system of the periparturient cow; such as mastitis, metritis, and other diseases. Preventing these diseases will necessitate identification of factors at the molecular (genome, transcriptome, and proteome) and whole animal (nutritional, hormonal, and management) level contributing to the immune suppression. With this knowledge we can develop strategies to circumvent these factors. In order to develop new control methods that are more effective and less reliant on use of antibiotics, it is necessary to understand the basic immunologic mechanisms of the cells of the

bovine immune system. In addition it will be critical to understand the interaction between the immune system and pathogens and how pathogens adapt and escape immune destruction.

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Vitamin-D in the Immune System: Genomic and Non-Genomic Actions. *Mini Rev Med Chem.* 2015; 15: 953-963. The ability of vitamin D to check and mediate the biology of calcium as a signal molecule, however, relates its function to energetics (a task particularly associated with mitochondria and ER) and to the inflammasome (and immunity), just at the cellular level. 36. Gottlieb R.A. Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and many other biological effects.[1][2] In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D2 (ergocalciferol).[1][2][3]. Vitamin D also affects the immune system, and VDRs are expressed in several white blood cells, including monocytes and activated T and B cells.[22] In vitro, vitamin D increases expression of the tyrosine hydroxylase gene in adrenal medullary cells, and affects the synthesis of neurotrophic factors, nitric oxide synthase, and glutathione.[23]. Vitamin D enhances intestinal calcium and phosphate absorption, stimulates osteoclast differentiation and calcium reabsorption from bone and promotes mineralization of the bone matrix. First evidence for the positive effect of vitamin D intake for human health came from early studies on rickets and osteomalacia (reviewed in [1]). immune system [7,8]. This suggests an important impact of vitamin D on a much wider aspect of human health than previously known. Early evidence that vitamin D acts as important stimulant for innate immunity came from reports about tuberculosis treatment with cod liver oil (reviewed in [39]). 145. Hart P.H., Gorman S., Finlay-Jones J.J. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat. Rev. Immunol.*, 2011, Vol. 11, pp. 584-596. 156. Hobaus J., Thiem U., Hummel D.M., Kallay E. Role of calcium, vitamin D, and the extrarenal vitamin D hydroxylases in carcinogenesis. *Anticancer Agents Med. Chem.*, 2013, Vol. 13, no. 1, pp. 20-35. 184. Đšhoo A.-L., Chai L.Y. A., Koenen H.J.P.M., Kullberg B.-J., Joosten I., van der Ven A.J.A.M., Netea M.G. 1,25-dihydroxyvitamin D3 modulates cytokine production induced by *Candida albicans*: impact of seasonal variation of immune responses. *J. Infect. Diseases*, 2011, Vol. 203, pp. 122-130. Numerous studies have demonstrated that the immune system of a dairy cow around the time of calving is suppressed. Beginning about 1 to 2 wk before calving and lasting until between 2 and 4 wk after calving, numerous immune functions have been shown to be inhibited during this time period (Kehrli et al., 1989). 1981. Vitamin D metabolites in plasma of cows fed a prepartum low-calcium diet for prevention of parturient hypocalcemia. *J. Dairy Sci.* 64:217-226.