

Interactions between Metabolic Disease and the Immune System: Why Cows Are Likely to Develop Mastitis at Freshening.

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Introduction

The transition from pregnant, non-lactating to non-pregnant, lactating is too often a disastrous experience for the cow. Most of the metabolic diseases of dairy cows - milk fever, ketosis, retained placenta (**RP**), and displacement of the abomasum - occur within the first two wk of lactation. In addition to metabolic disease, the majority of infectious disease experienced by the dairy cow, especially mastitis, but also diseases such as Johne's disease and Salmonellosis, become clinically apparent during the first two wk of lactation.

This review will make two assumptions before reviewing studies correlating metabolic disease with changes in infectious disease resistance. One of those assumptions is that mammary gland infections are less likely in animals with a *strong* immune system and that periparturient immune suppression exists and predisposes cows to mastitis and other infectious disease. This assumption helps explain why an animal that has harbored bacteria, within the udder or within the gut, for a long period without clinical disease suddenly breaks with the disease shortly after calving. The second assumption is that assays scientists have utilized to assess functional capacity and number of immune cells are reasonably accurate. The relative merit of the assays used in different laboratories will not be debated in this review. Excellent reviews are available which support these assumptions (Sordillo et al., 1997; Burvenich et al., 1994; Kehrl and Shuster, 1994; Tyler et al., 1993; Hogan et al., 1993; Senft and Neudecker, 1991). This review will examine the evidence that metabolic disease in cattle increases the susceptibility of the cow, particularly the periparturient cow, to development of infectious disease, with special emphasis on mastitis. Then each type of metabolic disorder will be examined

individually to discern the effect it might be having on the immune system.

Associations Between Metabolic Diseases and Mastitis

Several epidemiological studies have demonstrated that there is an association between the development of metabolic disease and subsequent development of mastitis. In a study of NY dairies (2,190 cows) there was a very strong association between parturient hypocalcemia or milk fever and mastitis. The odds ratio (multiplicative increases in occurrence) suggested that a milk fever cow was 8.1 times more likely to develop mastitis than a cow that had not had milk fever. The odds ratio for development of coliform mastitis was even greater (odds ratio, 9.0; Curtis et al., 1983). In a Swedish study, ketosis increased the risk of mastitis 2 fold (Oltenu and Ekesbo, 1994). A second larger study (18,110 Swedish Red and White cows in 924 herds and 14,940 Swedish Friesian cows in 772 herds) found that the risk of mastitis was increased in cows that had suffered a RP (Emanuelson et al., 1993). Twinning, dystocia, retained fetal membranes and lameness before first breeding service increased the risk of mastitis before first service in a ten herd study from England (Peeler et al., 1994).

Does Milk Production Affect Immune Status?

Neutrophil and lymphocyte function is diminished in the periparturient period, especially in the dairy cow (Kehrl et al., 1989 a,b). The onset of milk production imposes tremendous challenges to the mechanisms responsible for energy, protein, and mineral homeostasis in the cow. Negative energy,

protein, and/or mineral balance associated with the onset of lactation may be partially responsible for the immunosuppression observed in periparturient dairy cattle. Mastectomy of pregnant dairy cows removes the impact of milk production, while presumably maintaining endocrine and other changes associated with late pregnancy and parturition. Mastectomy would be expected to improve immune function in the periparturient dairy cow, if milk production is an immunosuppressive factor. Using ten mastectomized and eight intact multiparous Jersey cows (all intact cows developed milk fever) we assessed the ability of neutrophils to kill microbes, as assessed by neutrophil myeloperoxidase activity during the periparturient period (Kimura et al., 1999). Neutrophil myeloperoxidase activity decreased equally before parturition in both groups. While there was a quick recovery of neutrophil myeloperoxidase activity in mastectomized cows, there was no recovery in intact cows after parturition throughout the study, which lasted until d 20 postpartum. Lymphocyte production of gamma-interferon in vitro declined significantly at parturition in intact cows, but did not decrease significantly in the mastectomized cows. In intact cows, all T cell subset populations (i.e. CD3, CD4, CD8 and gamma-delta positive cells) decreased as a percentage of total PBMC at the time of parturition, while the percentage of monocytes increased. These population changes have previously been shown to be associated with the immune suppression commonly observed in periparturient cows. Mastectomy eliminated these changes in leukocyte subsets (Kimura et al., 2002). These results suggest: 1) the mammary gland may produce substances which directly affect immune cell populations or 2) metabolic demands associated with the onset of lactation negatively impact the composition of circulating PBMC populations. We will make the assumption that the 2nd possibility is the more likely. Two metabolic factors were greatly impacted by mastectomy. Mastectomy eliminated hypocalcemia at parturition. Plasma non-esterified fatty acid (NEFA) concentration rose dramatically in intact cows at calving and did not return to baseline level for > 10 d. In contrast NEFA concentration in mastectomized cow plasma rose only slightly at calving and returned to baseline level 1-2 days after calving.

It is clear that the intact cow mobilizes a much larger amount of body fat than does the mastectomized cow, suggestive of a severe negative energy balance at the onset of lactation.

At the risk of oversimplifying, the rest of this paper will focus on how disturbances in calcium metabolism (milk fever), energy metabolism (ketosis), and protein deficiency (ketosis, fatty liver complex), could cause increased susceptibility of the dairy cow to mastitis.

Hypocalcemia and Mastitis Susceptibility

Milk fever cows are at increased risk of developing mastitis (Curtis et al., 1983). Why?

Calcium is necessary for proper contraction of muscle. Severe hypocalcemia prevents skeletal muscle contraction to the point that the clinical syndrome known as milk fever occurs. Muscle contraction is reduced by any decrease in blood calcium, however it must be severe before we observe the *downer cow*. Daniel (1983) demonstrated that contraction rate and strength of the smooth muscle of the intestinal tract is directly proportional to blood calcium concentration. The teat sphincter consists of smooth muscle, which must contract if closure of the teat end is to occur. If low blood calcium reduces teat sphincter contraction, the teat canal may remain open inviting environmental pathogens to enter the mammary gland. We generally associate milk fever with the day of calving, but we have demonstrated that many cows remain subclinically hypocalcemic for the first week of lactation (Goff et al., 1996). Hypocalcemic cows tend to spend more time lying down than do normocalcemic animals. Again this could increase teat end exposure to environmental opportunists.

Immune cells need to be able to sense what is going on in their surroundings and react accordingly. For example, macrophages ingest foreign materials they encounter. Cell receptors react to ligand binding by converting this extracellular signal to an intracellular event – a process called signal transduction. Alteration of intracellular calcium concentration is a common second messenger of many immune cell receptor mediated responses. In some cases the receptor-

ligand interaction opens ion channels that allow extracellular calcium to enter the cytosol, raising intracellular calcium. Plant lectins that are used to stimulate T-cell division in vitro are known to open calcium channels in the cell membrane. A 50% decline in extracellular ionized calcium concentration, typical of the cow with milk fever, could slow the entry of extracellular calcium thru membrane ion channels thus blunting the response of the cell. Normally extracellular calcium concentration is around 10,000 fold greater than intracellular resting calcium concentration. Could a reduction to just 5,000 fold greater have major effects on the immune system? No work has been done in vivo to examine this possibility.

However, in most cases the receptor-ligand interaction activates protein kinases and phospholipases, which through a series of events (for example, production of inositol triphosphate) cause release of calcium to the cytosol from intracellular stores located in the mitochondria and endoplasmic reticulum. A rise in intracellular calcium is one of the signals required for T-cell activation. Kimura et al. (2001) used calcium sensitive dyes to monitor intracellular calcium concentration by flow cytometry. Peripheral blood mononuclear cells were isolated from 13 periparturient Jersey cows and stimulated (using CD3 monoclonal antibody or hydrogen peroxide). The intracellular calcium concentration was measured by ratiometric analysis of Fluo-4/Fura-red ratios by flow cytometry. Plasma calcium concentration reached a nadir at calving of about 6 mg/dl. Intracellular calcium response to stimuli tended to decrease at calving and was greatly increased following calving. Intracellular calcium store size was estimated by analysis of intracellular calcium increase following stimulation in the presence of media without calcium (i.e. no extracellular calcium) and in the presence of pervanadate and ionomycin, which block uptake of calcium back into mitochondria and endoplasmic reticulum. Intracellular calcium stores also reached a nadir at calving. These data suggest the hypocalcemia commonly observed in periparturient dairy cows reduces intracellular calcium stores decreasing the ability of the cells to respond to activating stimuli.

Another interesting study was conducted by Swierenga et al. (1976). Injection of sheep red blood cells into rats stimulates splenic DNA synthesis, which eventually results in a rise in

circulating antibody level. If they removed the parathyroid glands from rats, causing an acute hypocalcemia, 24 hr prior to injection of the sheep RBC the splenic cell proliferative response was greatly blunted. If they removed the parathyroid glands after the sheep RBC had been administered the response by the spleen cells was normal. These data suggested that parathyroid hormone and calcium could affect the immune response by affecting the proliferative phase of this response.

Hypocalcemia also acts as a stressor to the cow. Cows typically exhibit a 3-4 fold increase in plasma cortisol as part of the act of initiation of parturition. However subclinically hypocalcemic cows may have 5-7 fold increases in plasma cortisol on the day of calving and the typical milk fever cow may exhibit plasma cortisol concentrations that are 10-15 fold higher than pre-calving plasma cortisol concentration (Horst and Jorgensen, 1982). Cortisol is generally considered a powerful immune suppressive agent and likely exacerbates the immune suppression normally observed in the periparturient period. Exacerbates rather than causes because most studies suggest that immune suppression begins 1-2 weeks before calving (Kehrli et al., 1989 a, b; Ishikawa, 1987; Kashiwazaki et al., 1985), and the cortisol surge is fairly tightly confined to the day of calving and perhaps the day after calving.

Despite the strong epidemiological case and the evidence that intracellular calcium stores may be depleted in the hypocalcemic cow, we have not been able to demonstrate a more severe decline in immune function in the hypocalcemic periparturient cow than in the normocalcemic periparturient cow.

We fed ten older Holstein cows a high calcium, high cation prepartal diet to induce hypocalcemia (Kehrli and Goff, 1989; Kehrli et al., 1990). Five cows received daily parathyroid hormone injections for a few days before calving and in the first days of lactation to prevent any hypocalcemia from developing. The control cows did develop moderate hypocalcemia at calving and one of the five developed clinical milk fever. However, all cows experienced the same degree of leukopenia (attributable to an absolute and relative neutropenia) during the 1st wk after calving, decreased serum congenitinin activity during the first three wk postpartum, and decreased concentration of serum IgG1 during

the three wk before calving. All cows exhibited a similar severe loss of immune cell function in the weeks surrounding parturition. Neutrophil function (myeloperoxidase, native chemiluminescence, antibody-dependent cell-mediated cytotoxicity, cytochrome C reduction) and lymphocyte function (proliferative responses to lectins) were similar in hypocalcemic and normocalcemic cows. In general these functional assays required that the cells being examined be placed in culture media, which generally contained the same level of ionized calcium seen in normal cows – about 1.0 mM. Could these in vitro assay conditions have negated the effect hypocalcemia in vivo may have had on immune cell function? If we believe our assays gave us a true description of the effect of hypocalcemia we might conclude that the degree of hypocalcemia observed did not have a large or irreversible influence on neutrophil and lymphocyte function in periparturient cows. The epidemiological evidence of increased mastitis in cows developing milk fever must therefore primarily be the result of increased teat end exposure to environmental contamination.

Ketosis and Mastitis Susceptibility

Ketosis is diagnosed whenever there are elevated levels of ketones in the blood, urine, or milk of a cow. The disease is always characterized by a decline in blood glucose as well. In lactation, the amount of energy required for maintenance of body tissues and milk production exceeds the amount of energy the cow can obtain from her diet, especially in early lactation when dry matter intake is still low. As a result, the cow must utilize body fat as a source of energy. Every good cow will utilize body reserves in early lactation to help her make milk. However, there is a limit to the amount of fatty acid that can be handled and used for energy by the liver (and to some extent the other tissues of the body). When this limit is reached, the fats are no longer burned for energy but begin to accumulate within the liver cells as triglyceride. Some of the fatty acids are converted to ketones. The appearance of these ketones in the blood, milk, and urine is diagnostic of ketosis. As fat accumulates in the liver it reduces liver function - and a major function of the liver in the dairy cow is to produce glucose.

Recent work (Grummer, 1993) demonstrates the importance of feed intake at

calving on the etiology of the fatty liver-ketosis syndrome. On average, dry matter intake decreases by 20-30% one or two days before calving, and does not recover until one to two days after calving (Bertics et al., 1992; Marquardt et al., 1977). Interestingly, liver biopsies showed that liver triglycerides were increased 3-fold by the d of calving. Triglyceride buildup in the liver is a much earlier phenomena than previously assumed. Even more interesting; when cows were fitted with rumen fistulas and dry matter intake was not allowed to drop around the time of calving by forcing feed into the rumen, liver lipids and triglycerides increased only a small amount. Similar results were also achieved by daily drenching of cows with propylene glycol (1 L/d) during the week before and after calving (Studer et al., 1993).

The conclusion is that energy intake must not be compromised during the days around calving. Any factor restricting feed intake around calving (such as milk fever or RP) increases fat accumulation in the liver, affecting the energy deficit of the cow and increasing the risk of fatty liver-ketosis.

The fresh cow is also in negative protein balance shortly after calving. Generally this is not perceived to be as big a problem as the negative energy balance of early lactation, but the typical cow will lose 37 lbs body protein during the first two weeks of lactation. Much of this body protein is being used to support the amino acid and glucose requirements of milk production (Paquay et al., 1972). Therefore in many respects the dairy cow in early lactation is in a physiological state comparable to that of humans and rodents with prolonged protein-calorie restriction. Glutamine is the most abundant free amino acid in human muscle and plasma and is utilized at high rates by rapidly dividing cells, including leucocytes, to provide energy and optimal conditions for nucleotide biosynthesis. As such, it is considered to be essential for proper immune function. In humans, plasma glutamine is known to fall in patients with untreated diabetes mellitus, in diet-induced metabolic acidosis and in the recovery period following high intensity intermittent exercise (Walsh et al., 1998). Interestingly, plasma glutamine does not seem to be depressed in the dairy cow at calving – when body fat mobilization is rapidly increasing and presumably protein catabolism would also be

increasing rapidly. However plasma glutamine does decrease as the cow progresses into the early weeks of lactation (Zhu et al., 2000; Meijer et al., 1995).

Does the relative calorie-protein deficiency of the early lactation cow impact her immune response? In humans, protein-calorie restriction has severe effects on cell-mediated immunity. There is often widespread atrophy of lymphoid tissues and this can cause a 50% decline in the number of circulating T-cells. Surprisingly antibody responses are intact and phagocytosis of bacteria is relatively normal. However destruction of bacteria within the phagocytes is impaired (Roitt, 1991). Moderate calorie restriction (with or without a severe reduction in fat intake) has been used to ameliorate the effects of auto-immune disease, presumably by reducing cell-mediated immune responses (Leiba et al., 2001).

In mice, malnutrition leads to immune dysfunction with greatly increased morbidity. The macrophage is central to both immune effector and autoregulatory functions and is critical to host-defense mechanisms. Peritoneal macrophages were isolated from mice receiving ad lib or calorie restricted diets for 21 days. These restrictions were equivalent to a decrease in calorie intake of 21.9%. After stimulation of the cultured macrophages with lipopolysaccharide, supernatant prostaglandin E2 was determined and was significantly elevated in macrophages from the calorie-restricted mice compared with the ad-libitum-fed mice (Stapleton et al., 2001). Prostaglandin E2 is generally considered to be an immune suppressive agent, especially when released from damaged tissues (Roitt, 1991). Mice fed protein deficient diets and experimentally infected with parasites had worm burdens that were significantly higher than in mice fed adequate protein (Boulay et al., 1998). C57Bl/6 mice fed a low-calorie diet demonstrated a marked reduction in T-dependent-antigen-specific lymphocyte proliferation and antibody response when compared to mice fed ad libitum. The depressed lymphocyte response seen in calorie-restricted animals is attributed to a defect in both the macrophages and T cells in antigen processing, presentation, and/or proliferation (Christadoss et al., 1984).

We know very little about the effect protein/energy restriction might have on bovine

immune function. One small study examined the effect of protein/energy restriction for 133 days on nine cows infected with tuberculosis. Dietary restriction did not result in any significant reduction in skin sensitivity to PPD, in vitro production of IFN-gamma, or lymphocyte blastogenesis. The number of circulating BoCD4+ cells and B cells were similar in both the malnourished and the control cattle. However, significantly lower numbers ($P < 0.01$) of circulating BoCD2+ cells, BoCD8+ cells, WC1+ gamma delta T cells and ACT2+ cells were found in the malnourished cattle (Doherty et al., 1996).

There is some concern from studies done in human diabetics suffering from ketoacidosis that circulating ketone bodies may be affecting the immune system directly (Gregory et al., 1993). Several studies have looked at the effect addition of ketones and/or glucose to the media used to culture normal bovine leukocytes might have on their function. Franklin et al. (1991) examined the effects of beta-hydroxybutyrate, acetoacetate, acetone, acetate, butyrate, and glucose on in vitro lymphocyte proliferation of lymphocytes stimulated with concanavalin A, phytohemagglutinin-P, or pokeweed mitogen. Ketones, butyrate, and glucose at concentrations occurring in vivo had minimal effects on bovine lymphocyte proliferation in vitro. Only very high levels of beta-hydroxybutyrate inhibited proliferation. Nonnecke et al. (1992) examined the effect of ketones and glucose on in vitro lymphocyte immunoglobulin secretion. Results from experiments evaluating effects of glucose concentrations on IgM secretion indicated that plasma glucose concentration associated with the ketotic state (1.66 mM), compared with normal plasma glucose concentration (3.33 mM), did not affect total or antigen-specific IgM secretion. Adding a mixture of ketones to the culture media, which approximated plasma levels of severely ketotic cows, inhibited mitogen-induced IgM secretion in 11.1 mM glucose-supplemented cultures, but not in cultures with 3.33 or 1.66 mM glucose. High ketones and high plasma glucose may be more similar to the human ketoacidotic state, and could help explain the immune deficits observed in diabetics. These data indicate that effects of ketones and acetate on IgM secretion are dependent on the concentration of glucose in culture and suggest that changes in plasma glucose, ketone, and acetate concentrations associated with bovine ketosis do not alter IgM secretion in vivo.

Sartorelli et al. (2000), working with sheep determined that only beta-OH butyrate at concentrations seen in mild ketosis, could decrease bactericidal activity. Hoeben et al. (1997) found that butyrate at a level consistent with mild ketosis (1 to 2.5 mM) was able to reduce respiratory burst activity of bovine neutrophils. However it remains unclear how butyrate affects the cells.

A few studies have been done on lymphocytes isolated directly from normal cows and cows with clinical and subclinical ketosis and then placed into culture. Leukocytes of cows with clinical symptoms and the highest concentration of ketones and free fatty acids in blood responded with the lowest levels of interferons alpha and gamma to three interferon inducers: Newcastle Disease Virus, phytohemagglutinin and concanavalin A. Depression in interferon PHA stimulated synthesis correlated with a very low mitogenic response of blood lymphocytes. A correlation between the stage of ketosis and the level of interferon production in milk leukocytes was also observed (Kandefer-Szerszen et al., 1992).

Zerbe et al. (2000) examined the relationship between liver triacyl glycerol (**TAG**) content and immunophenotypical and functional properties of neutrophils of dairy cows in the peripartum period. Increased liver TAG content (> 40 mg/g), which was considered the upper level of normal, went in parallel with a reduced expression of function-associated surface molecules on blood neutrophils. Moreover, in cows with high liver TAG levels the antibody-independent and -dependent cellular cytotoxicity (**AICC**, **ADCC**) of blood PMN was markedly reduced. PMN also were less capable of reactive oxygen species generation after stimulation with phorbol myristate acetate.

What Is the Energy Cost to Mount an Immune Response?

Little to no work has been done to examine this issue in cattle. However, in humans suffering from severe infection causing sepsis (various degrees of fever, increased WBC count, and acute phase protein production), the resting energy expenditure (determined by indirect calorimetry), increased progressively over the first week of the infection to around 40% above normal and was still elevated three weeks from

the onset of illness. As an aside; over a 3-week period patients lost 13% of their total body protein (Plank and Hill, 2000).

No such measurements have been reported for cattle. However if we are allowed to extrapolate and speculate we can go through a few calculations. Maintenance energy for a 600 kg dairy cow is approximately 9.7 Mcal Net Energy / day. If the cow must also increase energy expenditure 40% to mount an inflammatory response, the energy requirement increases by nearly 4 Mcal / day. This is roughly equivalent to a requirement that the cow consume an additional 2.4 kg of diet (assuming a diet that provided 1.65 Mcal NE₁/kg). Can the periparturient cow, already in negative energy balance, be expected to successfully mount a rapid immune response? If she is in fact in negative protein balance as well, will her immune system produce the immunoglobulins and acute phase proteins necessary to fight an infection while it is still in the acute phase to prevent it from escalating to a clinical infection?

Mastitis and Retained Placenta

There has been some association between the development of RP and the incidence of mastitis (Emanuelson et al., 1993; Peeler et al., 1994). We have recently confirmed studies begun by Gunnink (1984) that suggest the two are likely linked because both are due to immune suppression in affected cows. Gunnink's theory suggested the fetal placenta must be recognized as *foreign* tissue and rejected by the immune system after parturition to cause expulsion of the placenta. We hypothesized that impaired neutrophil function causes RP. We examined the ability of neutrophils to recognize fetal cotyledon tissue as assessed by a chemotaxis assay, which utilized a placental homogenate obtained from a spontaneously expelled placenta as the chemoattractant. Neutrophil killing ability was also estimated by determining myeloperoxidase activity in isolated neutrophils. Blood samples were obtained from 142 periparturient dairy cattle in two herds. Twenty cattle developed RP (14.1 %). Neutrophils isolated from blood of cows with RP had significantly lower neutrophil function in both assays prior to calving and this impaired function lasted for 1-2 wk after parturition. Addition of antibody directed against interleukin-8 (**IL-8**) to the cotyledon preparation used as a chemoattractant inhibited

chemotaxis by 41% suggesting one of the chemoattractants present in the cotyledon at parturition is IL-8. At calving, plasma IL-8 concentration was lower in RP cows (51 ± 12 pg/ml) than in cows expelling the placenta normally (134 ± 11 pg/ml; Kimura et al., 2002). These data suggest neutrophil function determines whether or not the cow will develop RP and that depressed production of IL-8 may be a factor affecting neutrophil function in cows developing RP. This suppressed immune system could also explain why the same cows are more susceptible to mastitis. RP probably does not cause mastitis, but is symptomatic of a depressed immune system.

Conclusions

Periparturient immune suppression is a major factor contributing to the high incidence of infectious disease observed in dairy cows in early lactation. Metabolic diseases are generally a symptom of a body system gone haywire. When calcium metabolism is not working properly we are all aware that this affects the muscle strength of the animal. However it also has repercussions on the ability of the animal to resist infection. Similarly ketosis is a symptom of a severe energy imbalance in the animal. If there is not enough energy to support brain function, is it a surprise to find that it also affects immune cell function? We are only just now trying to determine which aspects of the immune system are affected by metabolic diseases. Obviously we must do all we can to improve calcium and energy status in the cow – utilizing the best nutritional programs and also considering such methods as force feeding of nutrients (drenching cows) at calving. Consideration should also be given to minimizing stress in newly calved cows. How many new social situations do you want this cow to experience? Studies suggest it takes up to 24 hrs for a cow to get back on full feed when she is moved into a new pen (she must find the new feed bunk and water trough, establish her place in the pecking order, etc.). Maternity pens should be clean and dry – the bigger the germ load the more work presented to an already challenged immune system.

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