

Recent Insights Into Ruminant Immune Function: Effects of Stress and Immunostimulatory Nutritional Products

*Neil E. Forsberg
Department of Animal Sciences
Oregon State University, Corvallis*

A. Introduction: General aspects of immune function

Ruminant livestock are continuously exposed to dangerous pathogens in their environment and, like other animals, rely upon a well-developed immune system to minimize likelihood of an infection. The immune system can be divided into two distinct (yet interacting) systems: **1**) the innate immune system and **2**) the adaptive or antibody-mediated immune system. Generally, we may view the innate system as the first line of defense against pathogens because several days are required to mount an antibody-response, especially to a new pathogen.

The innate immune system. The innate immune system consists of several interesting components: Aspects include:

1. Physical and chemical barriers to pathogens provided by epithelium, gastric acid and digestive enzymes
2. Cells which engulf and digest invading pathogens (e.g., neutrophils)
3. Receptors on the surface of these cells which recognize and bind to pathogens
4. Signaling molecules (e.g., chemokines, cytokines) which communicate sites of infection and regulate expression of immune genes

Neutrophils. Neutrophils are among the most important cells of the innate immune system. They are the first cell to arrive at a site of infection. In dairy cows, there are approximately 200 billion neutrophils of which about one-half are freely circulating in the blood (Burton and Erskine, 2003). The remainder are held in reserve in bone marrow where they are formed. Neutrophils express an extracellular binding protein on their membranes termed "L-selectin" (also termed CD62L). The role of L-selectin is to interact weakly with the endothelial cell wall thereby allowing the neutrophil to "roll" along the wall of a blood vessel and to "monitor" the cell wall for the presence of signals which indicate a local infection (Figure 1). The presence of pathogens in peripheral tissues causes release of local chemicals which then signal a rolling neutrophil of an infection. In response to these signals, L-selectin is shed from the surface of the neutrophil (see Figure 1) and other more adhesive molecules are expressed on its surface. These molecules essentially "glue" the neutrophil within the blood vessel adjacent to the site of infection. The activated neutrophil then migrates through the endothelial cell wall toward the invading pathogen. During migration, chemical signals

Contact: 112 Withycombe Hall, Corvallis, Oregon 97331-6702; (541) 737-1918 (office); (541) 737-1919 (FAX); neil.forsberg@orst.edu

originating from the site of infection (such as TNF α and interferon- γ) activate the neutrophil to become a mature “killer cell”. The mature neutrophil migrates toward the site of infection where it interacts with **pathogen-associated microbial patterns (PAMPs)** on the surface of pathogens via several types of receptors. These receptors are expressed on the surface of the neutrophil and include the following well-identified types (Figure 2):

- a- CD18 and CD14
- b- Toll-like receptors (TLRs)
- c- C3b and C3bi (complement factors)
- d- Fc

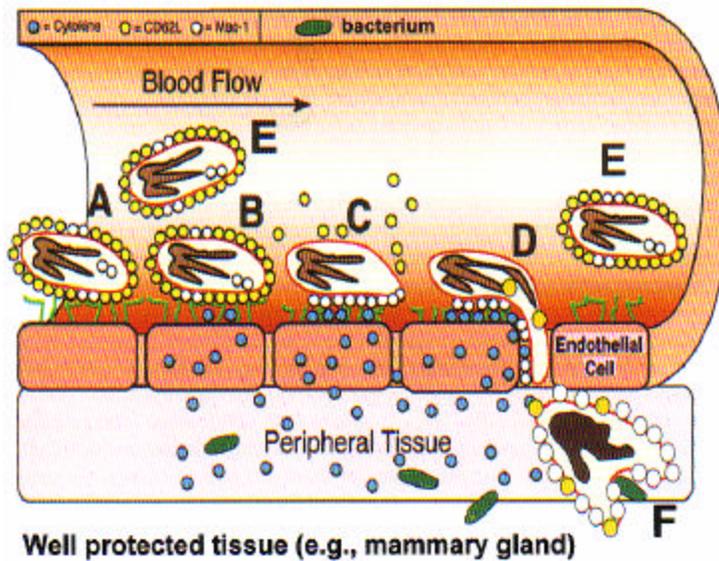
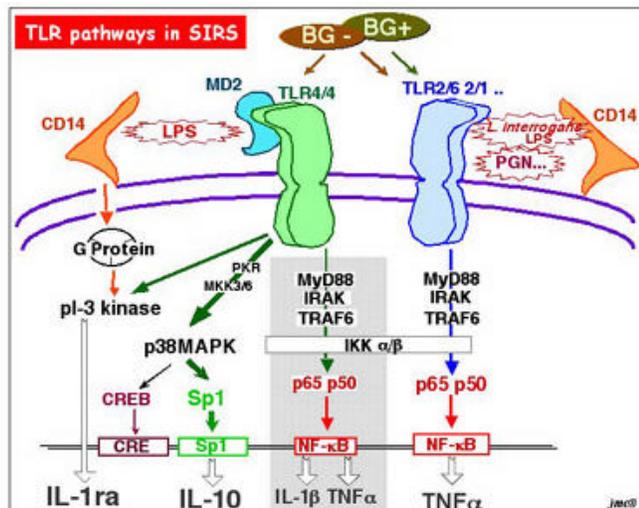


Figure 1 (above). Movement of neutrophils through a blood vessel. L-selectin (CD62L) is shown as circles on the surface of the neutrophil. These allow docking of the neutrophil with endothelium. Note shedding of L-selectin and migration of neutrophil into peripheral tissue toward a site of infection (F). Source: Burton and Erskine, 2003.

Figure 2. Toll-like receptors on surface of an immune cell and signal transduction following binding of TLRs with microbial PAMPs (Source: M. Adib-Conquy, C. Fitting, 2002).



Binding of neutrophils to pathogens via receptors. Both **CD14** and **CD18** receptors bind with lipopolysaccharide (LPS), a common polysaccharide structure associated with membranes of gram-negative bacteria. In addition, neutrophils express toll-like receptors (**TLRs**) which recognize and bind to additional structures associated with pathogens. So far, ten different toll-like receptors have been identified in mammals (Figure 2 and Table 1). TLRs play a critical role in early innate immunity to invading pathogens by sensing microorganisms. These evolutionarily conserved receptors recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (**PAMPs**: Invivogen, 2004). Stimulation of TLRs by PAMPs initiates a signaling cascade that involves a number of proteins, such as MyD88 and IRAK (Figure 2). This signaling cascade leads to the activation of the transcription factor NF- κ B which induces the secretion of cytokines that direct the adaptive (i.e., antibody-mediated) immune response. TLRs are predominantly expressed in tissues involved in immune function, such as spleen and peripheral blood leukocytes, as well as those exposed to the external environment such as lung and the gastrointestinal tract. Ten human and nine mouse TLRs have been characterized, seven of which have had their ligands identified. For examples, TLR2 is essential for the recognition of a variety of PAMPs, including bacterial lipoproteins, peptidoglycan, and lipoteichoic acids. TLR3 is implicated in virus-derived double-stranded RNA. TLR4 is predominantly activated by lipopolysaccharide. TLR5 detects bacterial flagellin and TLR9 is required for response to unmethylated CpG DNA (Table 1). Recently, TLR7 and TLR8 were shown to recognize synthetic antiviral molecules. These receptors are essential elements in host defense against pathogens by activating the innate immunity (Invivogen, 2004).

Bovine TLRs. Relatively few studies on PAMPs have been completed with **bovine** cells. So far, bovine immune cells have been reported to contain TLR2 and TLR4 (Werling et al., 2004). Polymorphisms have been reported in bovine TLR4 which may determine susceptibility to bovine respiratory disease and Johne's disease (White et al., 2003).

C3b and **C3bi** are components of the complement cascade whereas the **Fc** receptor binds to the “constant region” of antibodies. Hence, pathogens which are coated with complement factors or antibody (i.e., pathogens which are opsonized) are also recognized by activated neutrophils and are subsequently phagocytosed. In other words, activated neutrophils possess several means by which they recognize pathogens (Table 1).

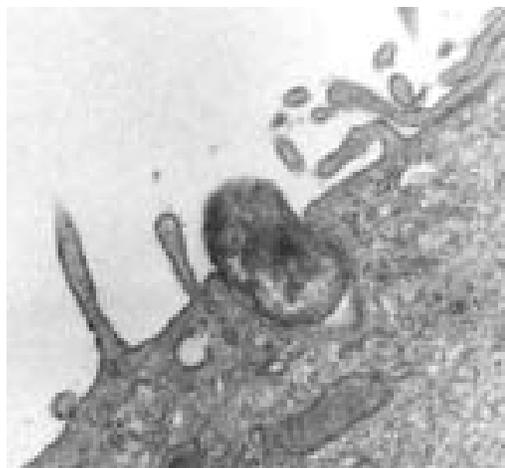
Phagocytosis and killing. The binding of neutrophils (and other phagocytic cells) to cell-surface markers of pathogens via these receptors then permits the phagocytic cell to engulf the invading pathogen and “kill” it (Figure 3). Presently, two mechanisms for “killing” are known. These include: 1) an oxidative burst, where the phagocyte expresses reactive oxygen species which destroy the phagocytosed pathogen, and 2) fusion of the engulfed pathogen with a lysosome-like structure to form a “phagosome”. The phagosome is rich in digestive enzymes which mediate complete digestion of pathogens.

Table 1. Summary of mechanisms by which neutrophils can recognize/bind to pathogen prior to phagocytosis

Neutrophil receptor	PAMP¹ or Ligand	Comment
CD14	lipopolysaccharide	direct binding to pathogen
CD18	lipopolysaccharide	direct binding to pathogen
TLR2	lipoprotein, peptidoglycan, lipotechoic acid	direct binding to pathogen
TLR3	virus-derived double-stranded RNA	direct binding to pathogen
TLR4	lipopolysaccharide	direct binding to pathogen
TLR5	flagellin	direct binding to pathogen
TLR7/8	Small synthetic anti-viral molecules	direct binding to pathogen
TLR 9	unmethylated CpG DNA	direct binding to pathogen
C3b/C3bi	complement factors	binds opsonized pathogen
Fc	“constant region” of antibodies	binds opsonized pathogen

¹ **PAMP: Pattern-associated molecular pattern**

Figure 3. In a process called phagocytosis, this macrophage cell engulfs a bacterium. Toll-like and other receptors direct phagocytes to recognize microbes. Note pseudopodial projections surrounding bacterium. Source: Travis, 2002.



B. Dairy cattle: Stress and infection

Common infections in dairy cattle. Mammary infections (mastitis) via microorganisms such as *Streptococcus uberis*, *S. agalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* are frequent on most dairy farms (Burton and Erskine, 2003). Mastitis/udder problems accounted for 27% of all cull decisions on dairies in the US in 2002 (NAHMS, 2003). Gastrointestinal infections resulting as secondary infections from acidosis or primary infections (*Salmonella*, *Clostridium*, coccidia and various molds including *Aspergillus fumigatus* and *Candida albicans*) also occur. For example, hemorrhagic bowel syndrome (**HBS**) appears to result from an intestinal infection and accounts for at least 2% of all dairy deaths (Kirkpatrick et al., 2001). However, on some dairies, deaths from HBS can reach levels near 10% of the entire herd/year. In all infections, the innate immune system plays a key initial role in fighting-off the initial immune challenge. The innate system is essential to allow the adaptive (antibody-mediated) system to “gear-up” and mount a more-specific and directed immune response.

An interesting challenge an animal has in fighting a mammary infection is related to the large dilution of immune cells which occurs when these cells enter the infected alveolus from the vasculature. As a result, the amounts of neutrophils which are secreted into infected mammary tissue are huge. The neutrophil is the primary immune cell entering alveolus to fight infections in ruminant animals. Concentrations of neutrophils in milk can reach levels as high as 4 million neutrophils/ml. In fact, neutrophils constitute the bulk of the somatic cell count (SCC). Rapid ability of a cow to “pump” neutrophils into the alveolar space at the initiation of an infection correlates strongly with disease resistance (Burton and Erskine, 2003).

Cooperation between the innate and acquired immune system in ruminants. Antibodies which are specific to an invading pathogen leak into the alveolar space (or other site of infection) to optimize clearance of a pathogen. Cows with a high titer against a specific antigen are able to deliver these antibodies into the milk alveolus via a leaky endothelium (arising from an inflammatory response). Arrival of reactive antibodies (i.e., IgG₂) in the alveolus coats (opsonizes) the pathogen and, as noted previously, allows neutrophil recognition of pathogens via Fc receptors (Table 1) and phagocytosis.

Stress and immune function. Some debate exists as to whether dairy cattle are “stressed” or not. There appears to be no question that dairy cattle are “stressed” during transition and this is at a maximum near time of parturition. However, it remains controversial whether lactating cows are physiologically stressed or not. One school of thought contends that high-producing dairy cows have been selected to manufacture high levels of milk and, as a result, are always close to metabolic disease. Hence, this school of thought believes that lactating cows are “stressed”. Others contend that high production, *per se*, is indicative of a lack of stress and of a healthy cow. In favor of the former belief, several studies have reported that a variety of normal production practices cause “stress”. Stressors in lactation may include a high energy diet (and potential acid

reflux), ketosis, milk fever, lameness, regular handling, post-partum stress, potential poor feeding practices, (Dobson and Smith, 2000), social isolation when sick animals are placed in a “hospital pen” (Boissy and LeNeindre, 1997) and artificial insemination (Nakao et al., 1994).

Mallard et al. (1998) reported that stress-associated immunosuppression is common in dairy cows and accounts for the high incidence of disease. Changes in both immune function and non-specific host defense mechanisms have been reported in dairy cows during transition and at onset of lactation (Ishikawa, 1987; Kashiwazaki et al., 1985; Kehrl et al., 1989; Gilbert et al., 1993; Guidry et al., 1976). Burton and co-workers at Michigan State University have identified one important mechanism by which stress brings about a reduction in immune function. Specifically, they have documented that glucocorticoids (i.e., cortisol) “spike” near parturition (Figure 4) and reduce L-selectin expression in neutrophils (Figure 5). This compromises one important aspects of an animal’s first line-of-defense against pathogen challenge. Specifically, a stressed, immunosuppressed animal has reduced ability to monitor endothelial cell lining for sites of infection and to attack and sequester pathogens. This may result in a “full-blown” infection (Figure 6).

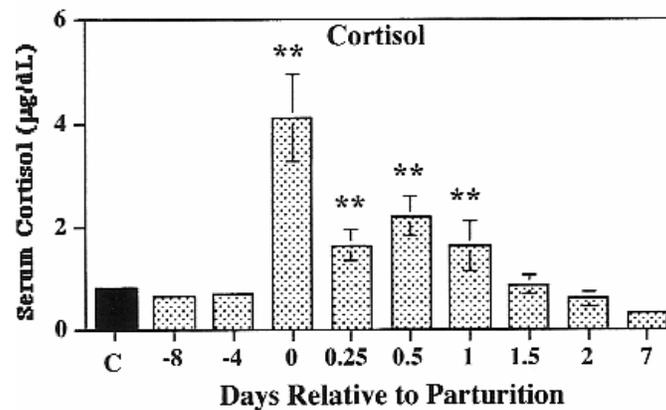


Figure 4 (above). Cortisol levels in dairy cattle relative to day of parturition. Note that cortisol peaks at day of parturition. Source: Weber et al., 2001.

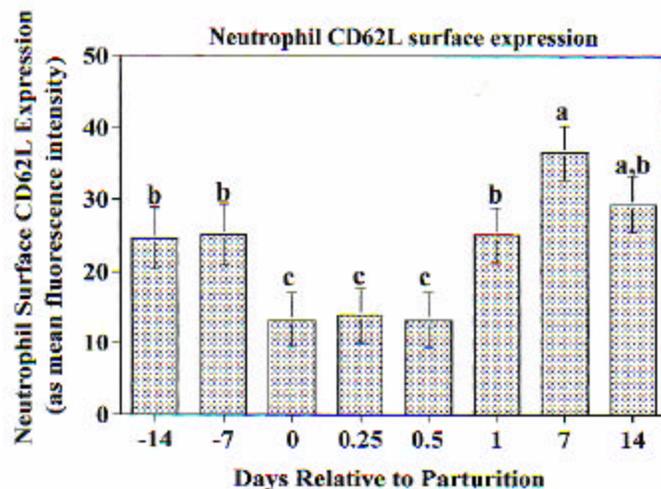
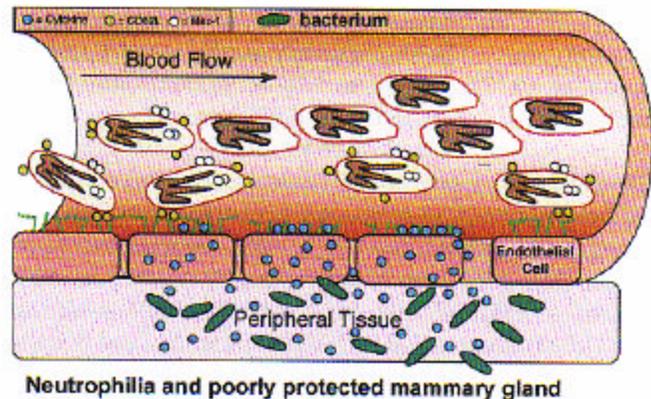


Figure 5 (right). Bars represent cow neutrophil L-selectin concentration relative to day of parturition. Source: Weber et al., 2001.

Figure 6. Neutrophils lacking expression of L-selectin (CD62L) in a stressed dairy cow (Source: Burton and Erskine, 2003).



C. Feed additives and regulation of immune function

Consumers and governments are becoming increasingly concerned about the use of antibiotics in livestock production vis-à-vis antibiotic resistance. At present, 22 million lbs of antibiotics are fed to livestock each year in the US (Feedstuffs, 2003). While incidents of antibiotic resistance and transmission of disease to humans have been established as a result of feeding livestock antibiotics, the cases remain sporadic and the risk to individual consumers remains very low. The National Research Council and Institute of Medicine have concluded that there is not yet sufficient evidence to declare that the practice of feeding sub-therapeutic levels of antibiotics to livestock constitutes an immediate public-health concern. However, the report cautions, "additional data might alter this conclusion" (NRC, IOM, 1999). Despite the lack of strong evidence that antibiotic feeding to livestock is an imminent threat to human health, the EU has banned many antibiotics for livestock and many US trading partners are beginning to ban importation of meat products raised with certain antibiotics. There is also a voluntary trend among large American food establishments toward selection of products which are raised "antibiotic-free". These developments place an imperative upon the industry to develop alternatives to large-scale antibiotic use.

New knowledge in mechanisms of immune function has created opportunities for scientists to design nutritional products which have potential to regulate, possibly even augment, the immune system. These products, if efficacious, could reduce reliance upon antibiotic use. This is not a particularly new concept because it has been known for decades that nutrition and immune function are linked. What is now possible is our ability to intelligently choose feed additives and components and also to specifically assess mechanisms by which nutritional products influence immune function.

We (Wang et al., 2004) designed a study to determine the effects of a commercial nutritional product (OmniGen-AF: Prince-Agri Products, Quincy, IL) on immune function in sheep. We allotted 60 growing (male and female) lambs to the following five treatments:

1. Control
2. Immunosuppressed
3. Immunosuppressed with daily intake of the nutritional product
4. Immunosuppressed with mold challenge
5. Immunosuppressed with mold challenge and daily intake of the nutritional product

Immunosuppression followed the model developed at Michigan State University where Azium (Dexamethasone: 0.1 mg/kg BW/day, twice/day) was administered intermittently for a period of 28 days (a model of extreme stress). Specifically, for treatments 2-5, Azium was administered via sub-cutaneous injection on 4 of 7 days of each week during the 4-week trial. The nutritional product was added to a dairy-type diet (i.e., high energy, alfalfa-based, rich in corn) at a level of 0.5% (w/w). The mold challenge for Treatments 3 and 5 consisted of addition of heavily-molded wheat mill run (WMR) which had been recovered from a Washington dairy which was experiencing high rates of hemorrhagic bowel syndrome. We determined that the mold infecting the WMR was *Aspergillus fumigatus*, a common mold with potential to cause ruminant mycoses (Sarfati *et al.*, 1996; Puntenney *et al.*, 2003). Blood samples were taken on a weekly basis and neutrophils were purified using Percoll gradient. L-selectin and interleukin-1 β (IL-1 β) concentrations in purified neutrophils were assessed by Western blotting using antibodies specific to these proteins.

We found that all animals receiving daily injections of Azium (i.e., immunosuppressed animals) grew more slowly ($P < 0.05$; Figure 7). These data demonstrate effects of extreme stress on growth.

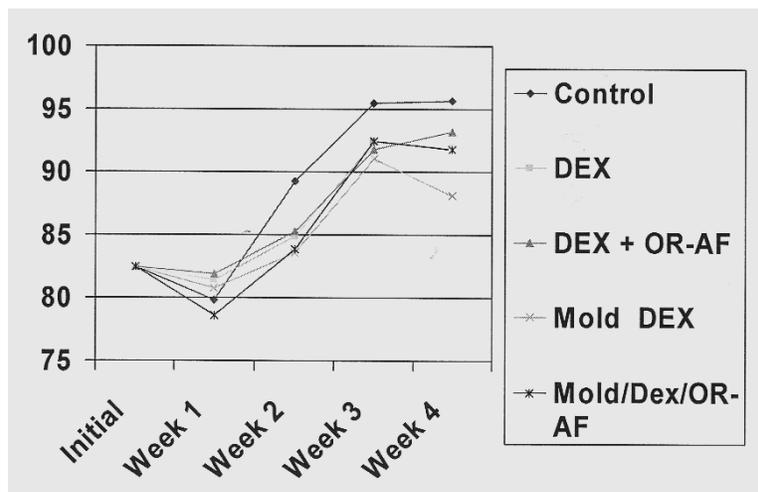


Figure 7. Body weights (pounds) of sheep on five treatments. Note that all animals receiving DEX (Azium) had reduced body weights relative to control sheep. The plateau in growth in Weeks 3-4 coincided with a change to cold, rainy weather.

We also found that Azium caused a marked reduction in both L-selectin (Figure 8a) but completely eliminated IL-1 β (Figure 8b). Addition of the nutritional product

(OmniGen-AF) to diets, whether in the presence of absence of mold, increased L-selectin markedly ($P < 0.05$). IL-1 β was increased slightly when OmniGen-AF was added to the regular diet; however, the addition of OmniGen-AF to the diet caused a marked increase in IL-1 β ($P < 0.05$) when *A. fumigatus* was present.

Implications. The feed product has been fed successfully nationally within the dairy market. Completion of this study in sheep suggests that one mode of action includes restoration of innate immune function (as indicated by restoration of neutrophil L-selectin and IL-1 β). Experiments are underway to determine additional mechanisms-of-action.

Additional observations. During the course of the study, three sheep (out of 12) in Treatment 4 (i.e., those receiving Azium and feed-borne mold) developed pyrexia. Of interest, this was the treatment group which showed a marked reduction in weights during Week 4 of the study (Figure 7). Rectal temperatures in these three sheep all exceeded 104.5 °C. These three animals appeared bloated, had ruminal atony and were lethargic. Veterinary analysis of the sheep indicated pneumonia. It was of interest (and possibly predictable) that immunosuppressed animals would develop an illness. We euthanized two of these sheep and found one with acidotic lesions on the ventral rumen and also detected an *A. fumigatus* fungal lesions lying in the center of the acidotic lesion. *A. fumigatus* DNA was found associated with the lymph node draining the ventral rumen, in blood and in liver. Evidently, in this animal, the “dairy diet” had caused acidosis and its immune system was inadequate to fight an invasive mycoses from developing (Figure 9).

These data are of considerable interest because they are the first to document ability of a designed nutritional product to restore normal immune function in immunosuppressed animals.

D. Summary

The livestock industry, since the 1950s, has relied upon sub-therapeutic levels of antibiotics to enhance animal growth and to prevent opportunistic infections. The practice is declining in Europe and other countries and could decline in the US as well. Knowledge of how pathogens signal the immune system to mount an immune response has created opportunities for scientists to design nutritional products which may mimic a pathogen challenge and restore immune function in immunosuppressed animals. We have tested a new generation nutritional product for its ability to restore immune function in immunosuppressed animals and find that two key indexes of immunity (neutrophil L-selectin and interleukin-1 β) respond quickly (< 1 week, data not shown). We predict new opportunities exist to maximize productivity of livestock with nutritional products.

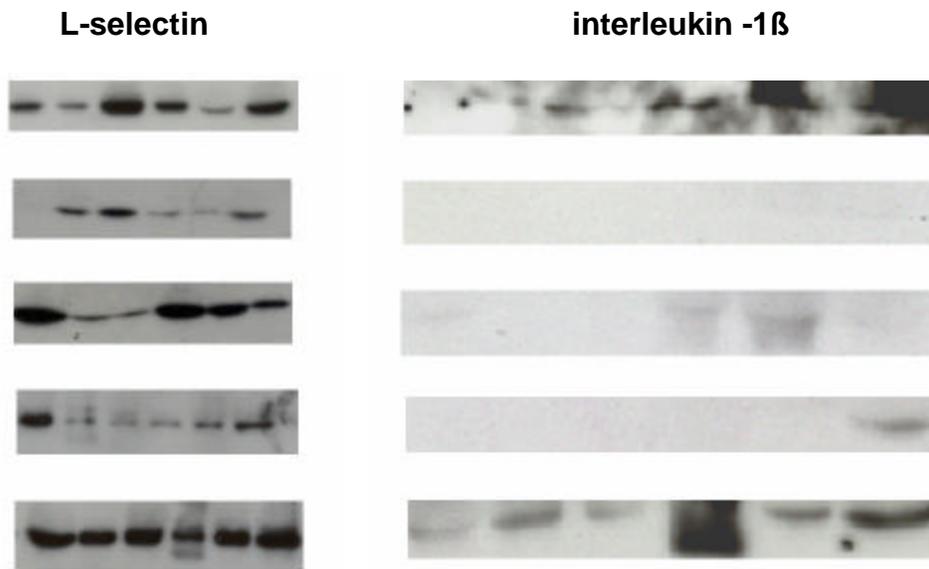


Figure 8 (above). Western blot analysis showing effects of the five experimental treatments on L-selectin (Left Panel) and IL-1 β (Right Panel). Treatments in each panel are arranged in a descending order from Treatments 1-5, respectively. Data are from Wang *et al.*, 2004 and are unpublished.



Figure 9 (left). Large (20 cm X 20 cm) ruminal lesion (about 2 weeks old) containing an *A. fumigatus*-positive lesion in an animal receiving Azium and *A. fumigatus*. Note hemorrhagic foci throughout the acidotic lesion.

Acknowledgements: We are grateful to the Oregon State University Sheep center for their support of this project.

Color illustrations. Many of the photographs in this proceedings paper are in color. However, they are not reproduced in color here. Color photographs of each photo are available by contacting the author or by accessing the Florida Ruminant Nutrition Symposium Website.

Conflict-of-interest disclosure: Neil Forsberg has participated in the development of technologies relating to OmniGen-AF and has a relationship with OmniGen Research LLC, a company which has licensed rights to distribute OmniGen-AF to Prince-Agri Products (Quincy, IL).

References

- Adib Conquy, M., C. Fitting. Immunological status of cardiac arrest and resuscitated patients. <http://www.pasteur.fr/recherche/RAR/RAR2002/Cytoinf-en.html>, 2002.
- Boissy, A., P. LeNeindre. Behavioral, cardiac and cortisol responses to brief peer separation and reunion in cattle. *Physiol. Behav.* 61:693-699, 1997.
- Burton, J.L., R.J. Erskine. Immunity and mastitis. Some new ideas for an old disease. *Vet. Clin. Food Anim.* 19:1-45, 2003.
- Dobson, H., R. Smith. What is stress, and how does it affect reproduction. *Anim. Reprod. Sci.* 60-61:743-752, 2000.
- Feedstuffs. AHI survey shows stable animal antibiotic use. Dec. 22, 2003, Page 4.
- Gilbert, R., Y. Grohn, P. Miller, D. Hoffmann. The effect of parity on peripartum neutrophil function in dairy cows. *Vet. Immunol. Immunopath.* 36:75-82, 1993.
- Guidry, A., M. Paape, R. Pearson. Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. *Am. J. Vet. Res.* 37:1195-1200, 1976.
- Invivogen. <http://www.invivogen.com/genedescription/TLR01.htm>, 2004.
- Ishikawa, H. Observation of lymphocyte function in perinatal cows and neonatal calves. *Jpn. J. Vet. Sci.* 49:469-475, 1987.
- Kashiwazaki, Y., Y. Maede, S. Namioka. Transformation of bovine peripheral blood lymphocytes in the perinatal period. *Jpn. J. Vet. Sci.* 47:337-339, 1985.
- Kehrli, M., B. Nonnecke, J. Roth. Alterations in bovine peripheral leukocytes during the prepartum period. *Aust. J. Vet. Res.* 50:215-220, 1989.
- Kirkpatrick M.A., L.L. Timms K.W. Kersting *et al.* Case Report – jejunal hemorrhage syndrome of dairy cattle. *Bov. Prac.* 35:104-115, 2001.
- Mallard, B., J. Dekkers, M. Ireland, K. Leslie, S. Sharif, C. Vankampen, L. Wagter, B. Wilkie. Alteration in immune responsiveness during the peripartum period and its ramifications on dairy cow and calf health. *J. Dairy. Sci.* 585-595, 1998.
- NAHMS (APHIS) 2003b. http://www.aphis.usda.gov/vs/ceah/cahm/Dairy_Cattle/Dairy02/Highlights-of-NAHMS-Dairy-2002.pdf
- Nakao, T., T. Sato, M. Moriyoshi, K. Kawata. Plasma cortisol levels in dairy cows to vaginoscopy, genital palpation per rectum and artificial insemination. *Zentralbl. Veterinarmed A.* 41:16-21, 1994.

- National Research Council (NRC) and Institute of Medicine (IOM: 1999). The use of drugs in food animals. Benefits and Risks. National Academy Press. Washington, DC.
- Puntenney, S., Y. Wang, N.E. Forsberg. Mycotic infections in livestock: Recent insights and studies on etiology, diagnostics and prevention of Hemorrhagic Bowel Syndrome. Proc. S.W. Anim. Nutr. Manag. Conf. 49-62, 2003.
- Sarfati, J., H. Jensen, J. Latge. Route of infections in bovine aspergillosis. J. Med. Vet. Mycol. 34:379-383, 1996.
- Travis, J. Biologists reveal the proteins that first see dangerous microbes. Week of Sept. 8, 2001 Vol. 160, No. 10.
- Wang, Y.Q., S. Puntenney, N.E. Forsberg. Immunoregulation in growing sheep. Submitted, 2004.
- Werling D., J.C. Hope, C.J. Howard, T.W. Jungi. Differential production of cytokines, reactive oxygen and nitrogen by bovine macrophages and dendritic cells stimulated with Toll-like receptor agonists. Immunology 111:41-52, 2004.
- White S.N., K.H. Taylor, C.A. Abbey, C.A. Gill, J.E. Womack. Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. Proc. Natl. Acad. Sci. U S A. 100:10364-10369, 2003.
- Weber, P.S.D., S.A. Madsen, G. W Smith, J.J. Ireland, J.L. Burton. Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet. Immunol. Immunopath. 83:213-240, 2001.