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IDENTIFICATION OF THE MECHANISMS BY WHICH OMNIGEN-AF, A NUTRITIONAL SUPPLEMENT, AUGMENTS IMMUNE FUNCTION IN RUMINANT LIVESTOCK

Y.-Q. Wang, S.B. Puntenney, and N.E. Forsberg[1]
Immunobiology Laboratory
Department of Animal Sciences
Oregon State University, Corvallis, OR 97331-6702

ABSTRACT: OmniGen-AF, a recently-developed nutritional supplement, has been reported to effectively improve health and performance of lactating dairy cattle. However, its mechanisms of action have not been fully characterized. The hypothesis of this research was that feeding immunocompromised ruminant livestock with OmniGen-AF increases innate immune function. To test this hypothesis, sixty sheep were assigned to five treatments: **1:** control, **2:** immunosuppressed with dexamethasone injection, **3:** immunosuppressed with OmniGen-AF, **4:** immunosuppressed with a pathogen challenge (*Aspergillus fumigatus*) and **5:** immunosuppressed with pathogen challenge and OmniGen-AF. Sheep were given these treatments for 28 days after which two indexes of innate immune function were assessed: **A:** L-selectin and **B:** interleukin-1ß (IL-1ß). Dexamethasone injection reduced innate immune function ($P<0.05$; i.e., reduced neutrophil L-selectin and IL-1ß concentrations). Administration of OmniGen-AF to non-pathogen-challenged sheep increased ($P<0.05$) L-selectin but did not affect IL-1ß ($P>0.05$). Pathogen challenge did not affect ($P>0.05$) L-selectin of IL-1ß. However, exposure to pathogen potentiated actions of OmniGen-AF on IL-1ß. Specifically, OmniGen-AF restored ($P<0.05$) normal levels of neutrophil L-selectin and boosted levels of IL-1ß in pathogen-challenged sheep. OmniGen-AF functions, in part, by augmenting innate immune function in immunosuppressed livestock.
Key Words: Immunity, Sheep, OmniGen-AF, L-selectin, Interleukin-1ß

Introduction

Animals are able to resist invasion by pathogens via a combination of the innate immune system and the adaptive (antibody-mediated) immune system (Janeway *et al*., 2001). The innate immune system represents the first line of defense and thereby provides the adaptive system the time it requires to develop an antibody response. Aspects of the innate system include physical epithelial barriers, gastric acid, digestive enzymes and activities of phagocytic cells which may be recruited to any site of infection (Janeway *et al*., 2001).

The neutrophil is a phagocytic cell of the innate immune system. It is manufactured in bone and released to circulate in blood where it functions to monitor for sites of an infection (Burton and Erskine, 2003). Neutrophils monitor sites infection by "rolling" along the endothelial lining of blood vessels where local production of cytokines causes them to firmly attach adjacent to a site of infection, to migrate through the endothelial lining toward pathogens and to subsequently engulf pathogens via phagocytosis (Burton and Erskine, 2003). Following ingestion, neutrophils digest pathogens via one of two mechanisms: an oxidative burst or via engulfment by lysosomes. Of interest, a recent study showed that neutrophils also cast bacteriocidal "nets" extracellularly. These "nets" consist of DNA and a mixture of proteases which digest pathogens (Brinkmann *et al*., 2004).

Neutrophil rolling is mediated by weak interactions between an extracellular neutrophil adhesion molecule (L-selectin) and other adhesion molecules associated with the endothelial cell surface (Burton and Erskine, 2001). L-selectin is critical to this process and recent studies have documented that glucocorticoid-mediated stress and immunosuppression bring about a reduction in innate immune function by causing release of the extracellular pool of neutrophil L-selectin (Tempelman *et al*., 2002; Weber *et al*., 2001). This thereby reduces of eliminates the ability of neutrophils to search for pathogens and enhances the likelihood of an infection. An example of this occurs at parturition in dairy cattle; a time when cows are maximally susceptible to infections such as mastitis (Burton and Erskine, 2003. The glucocorticoid (stress-related) peak which occurs at parturition brings about a marked reduction in neutrophil L-selectin and increases susceptibility to infection (Burton and Erskine, 2003). Similarly, neutrophil function is also supported by a variety of other molecules including the pro-inflammatory cytokine: interleukin-1ß (IL-1ß). IL-1ß is an inducible cytokine which plays critical roles in the neutrophil's ability to communicate with other cells and to mediate pathogen sequestration (Janeway *et al*., 2001).

To support animals' innate abilities to resist pathogen, producers often provide sub-therapeutic antibiotics in feeds. This is a common practice in North America with about 20-25 million lbs of antibiotics administered to livestock annually (UCS, 2003). However, the practice has fallen into disfavor in the European Union and some Asian countries (e.g., Japan) and there has been interest in developing alternatives to this practice as a strategy to possibly reduce the development of antibiotic-resistant human pathogens.

In this experiment, we tested the ability of a new feed product (OmniGen-AF) to augment innate immune function in ruminant livestock. Early reports have indicated its ability to inhibit fungal growth *in vitro* (Puntenney *et al*., 2003); however, field reports indicated it was effective in preventing diseases in lactating dairy cattle. To test this hypothesis, a sheep study was completed in which efficacy of OmniGen-AF was evaluated in immunosuppressed sheep and in immunosuppressed sheep challenged with pathogen (*A. fumigatus*).

Materials and Methods

This study was approved by Oregon State University's Animal Care and Use Committee. Sixty Polypay X Friesan/Suffolk sheep (25 wethers and 35 ewe lambs: ca. 85 lbs each) were assigned to five treatments in January, 2003. The treatments consisted of: **1** control, **2** immunosuppressed, **3** immunosuppressed with OmniGen-AF, **4** immunosuppressed and pathogen-challenged and **5** immunosuppressed, pathogen-challenged and supplemented with OmniGen-AF. Five males and seven females were randomly assigned to each treatment. The duration of the experiment was 28 days. Immunosuppression was induced by twice daily injection of dexamethasone (Azium: 0.1 mg/kg BW, twice/day). This is a model of extreme stress which was reported by Dr. Jeanne Burton at Michigan State University (Weber *et al*., 2001). Azium was injected sub-cutaneously in the neck region. OmniGen-AF® (Prince-Agri Products, Quincy, IL) was administered to animals in Treatments 3 and 5 by feeding at a dose of 0.5% w/w. Pathogen challenge was provided to Treatments 4 and 5 by daily administration of highly-molded wheat mill run (1 lb/head/day). This sample of wheat mill run had been obtained from a dairy in Washington State with high incidence of hemorrhagic bowel syndrome and abortions. Treatments 1, 2 and 3 were provided additional wheat bran (1 lb/head/day). All animals were fed alfalfa hay free choice and provided 0.75 lbs of ground corn/head/day. Sources of ground corn and wheat bran were baked at 95 ^{o}C for 24 hours prior to feeding to reduce the likelihood of introducing viable pathogen to animals on Treatments 1, 2 and 4.

Jugular blood samples (10 ml) were taken from animals on Day 28 of the study using citric acid as an anti-coagulent. Blood samples were immediately placed on ice and transported to the laboratory where neutrophils were isolated by Ficoll-Paque Plus (Amersham Biosciences) using gradient centrifugation according to manufacturer's recommendations.

Protein from neutrophils (20 µg) was applied to SDS-PAGE gels from six randomly-chosen animals on each treatment and electrophoresed. Samples were then transferred to a nitrocellulose membrane via Western blotting (Ausubel *et al*., 1989) and membranes were blocked with 5% skim milk in TTBS. Primary antibody (L-selectin or IL-1ß: VMRD, Pullman, WA) was exposed to membranes for 1 hour after which membranes were exposed to secondary antibody (goat anti-mouse HRP: BioRad) for 1 hour. Membranes were washed five times with TTBS and then exposed to Xray film for 1 hour (L-selectin) or overnight (IL-1ß) and developed. Intensity of exposures was determined by scanning densitometry using a BioRad VersaDoc 1000 imager and PDQuest software.

Animal Health. During the study, animal health was closely monitored. Rectal temperatures were taken weekly. When animals appeared ill, a veterinarian from the Oregon State University College of Veterinary Medicine was employed to diagnose illnesses.

Following completion of this study, animals were held in the Oregon State University sheep facility for a 28-day clearance period. This was judged by the OSU College of Veterinary Medicine to provide adequate time for clearance of residual Azium.

Statistical analyses. Concentrations of L-selectin and IL-1ß were expressed as arbitrary densitometer units. Analysis of a variance was used to determine if differences ($P<0.05$) existed among the five treatments (Steel and Torrie, 1980) using SAS. A Student-Neuman-Keul multiple range test was then used to test for individual treatment differences. A 5% level of significance was adopted for all comparisons.

Results and Discussion

Administration of Azium to Treatments 2, 3, 4 and 5 caused marked immunosuppression. Evidence of this is illustrated in both Figures 1 and 2 where Azium injection caused a marked reduction ($P<0.05$) in L-selectin concentration (Figure 1) and completely abolished ($P<0.05$) neutrophil IL-1ß (Figure 2; Treatment 2 compared to Treatment 1 [control]). These observations indicate clearly that the Burton model (Weber *et al*., 2001), which was developed as an model of extreme stress in dairy cattle, provides an adequate model for immunosuppression in sheep. Further work in this area may be directed toward titration of an optimal dose for immunosuppression in sheep.

During the 28-day study, few health problems were detected. However, in the fourth week of the study, three sheep on Treatment 4 (immunosuppressed and pathogen-challenged) developed lethargy, ruminal hypomotility and pyrexia (each over 40 ^{o}C). Mean body temperatures for all sheep are shown in Figure 3. The attending veterinarian diagnosed the animals as having pneumonia. Two of these three animals were slaughtered in the OSU College of Veterinary Medicine following the trial by a veterinary pathologist. One was devoid of any gastrointestinal pathology. The other demonstrated moderate but locally extensive ulcerative rumenitis with mild, emphysematous lymphadenopathy. Other animals on this treatment were not euthanized. It is possible that the pneumonia and gastrointestinal pathologies noted in one sheep were

related to the immunosuppressive effects of Azium and the presence of food-borne pathogen.

Effects of OmniGen-AF on indexes of innate immune function in non-pathogen-challenged sheep are shown also in Figures 1 and 2 (Treatment 3 versus Treatment 2). Addition of OmniGen-AF to the diet restored normal levels of L-selectin ($P<0.05$) but had no effect ($P>0.05$) on neutrophil IL-1ß concentration. These data indicate potential for the feed product to restore ability of neutrophils to monitor the endothelial cell lining for sites of infection but also indicate that IL-1ß function remains repressed, even in the presence of OmniGen-AF.

Animals on Treatments 4 and 5 were also challenged with a contaminated feed product (wheat mill run). We tested this feed and determined that it was contaminated with *A. fumigatus* (>1 million spores/g); however, the multitude of colors present in the feed indicated other molds were also present. Addition of OmniGen-AF to pathogen-challenged sheep also increased L-selectin ($P<0.05$) concentration (Figure 1). Of interest, the presence of pathogen potentiated actions of OmniGen-AF on IL-1ß. Specifically, OmniGen-AF caused a 2- to 3-fold increase ($P<0.05$) in IL-1ß concentration when fed in the presence of a heavily-molded feed sample.

It is difficult to envision how a nutritional product might augment immune function in the manner described here. However, it has been reported that nutrients have profound effects on immune status. Nutrients that have been demonstrated (in either animal or human studies) to be required for the immune system to function efficiently include essential amino acids, the essential fatty acid linoleic acid, vitamin A, folic acid, vitamin B6, vitamin B12, vitamin C, vitamin E, Zn, Cu, Fe and Se (Calder and Kew, 2002). Practically all forms of immunity may be affected by deficiencies in one or more of these nutrients. Undernutrition leading to impairment of immune function can be due to insufficient intake of energy. In this sense, OmniGen-AF, as a nutritional product, may exert its effects on immune function as do other nutrients.

Implications

Feed products such as OmniGen-AF have potential to augment innate immune function in ruminant livestock and, possibly, to improve herd health. Further work on abilities of this and similar products to replace a portion of the antibiotics currently fed at sub-therapeutic levels needs further investigation.

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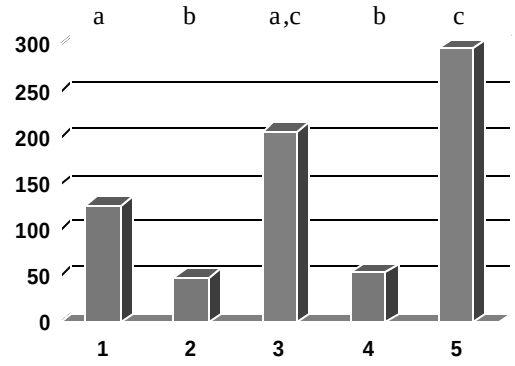

Figure 1. Neutrophil L-selectin concentrations in sheep exposed to the five experimental treatments outlined in text of article. Numbers on the *x*-axis correspond to Treatment. Numbers on the *y*-axis correspond to L-selectin in arbitrary densitometry units. Values are means of six animals following 28 days of treatment. If treatments do not share a common letter superscript, they different significantly (P<0.05).

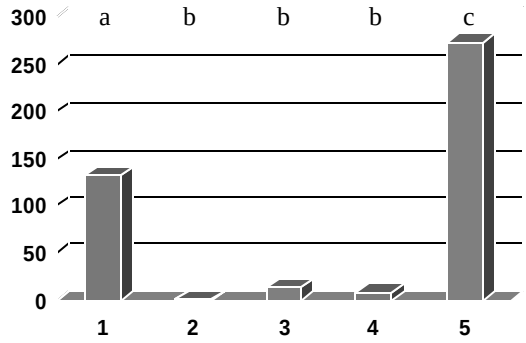

Figure 2. Neutrophil interleukin-1ß concentrations in sheep exposed to the five experimental treatments outlined in text of article. Numbers on the *x*-axis correspond to Treatment. Numbers on the *y*-axis correspond to IL-1ß in arbitrary densitometry units. Values are means of six animals following 28 days of treatment. Statistically-significant differences (P<0.05) are indicated in text. If treatments do not share a common letter superscript, they different significantly (P<0.05).

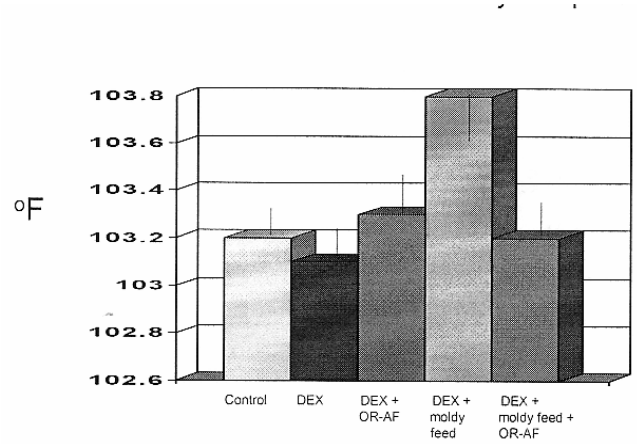

Figure 3. Effects of the five treatments on body temperatures of the sheep. Values are means of twelve sheep/treatment +/- SEM. No differences (P>0.05) were detected among treatments.