The development of a dexamethasone challenge model for evaluating feed additives in sheep

Austin J. Lea, MAS*; Benjamin R. Trible, PhD; Daniel S. Grum, PhD; Alysta D. Sewell, PhD; Jason R. Sewell, PhD; Murali M. Raghavendra Rao, PhD

Purina Animal Nutrition LLC, Gray Summit, MO

*Corresponding author: Austin J. Lea (ajlea@landolakes.com)

OBJECTIVE
To develop an accessible ruminant immune challenge model for rapid in vivo assessments of feed additives.

ANIMALS
60 hair-breed ram lambs.

METHODS
Sheep were randomly assigned to 1 of 4 treatments: treatment 1, not immunosuppressed, control fed (n = 12); treatment 2, immunosuppressed, supplemented with a yeast and botanical extract (n = 18); treatment 3, immunosuppressed, supplemented with a blend of natural aluminosilicates and yeast components (n = 18); and treatment 4, immunosuppressed, control fed (n = 12). Twice-daily injections of dexamethasone (Dex; 0.1 mg/kg bodyweight, SC) were used to induce immunosuppression throughout the study (from September 25, 2020, to November 2, 2020). All sheep were immunized with keyhole limpet hemocyanin (KLH) on days 0 and 14 and injected with heat-aggregated KLH, ID, to induce a skin induration on day 15. Measurements included body weight (BW), average daily gain (ADG), CBC, and skin induration diameter.

RESULTS
Dex treatment resulted in reduced BW and ADG that was not mitigated by either feed additive. Dex reduced lymphocyte percentage, RBC count, hemoglobin, hematocrit, and skin induration diameter and increased concentrations of granulocytes and granulocyte percentage. Effects on hematocrit, hemoglobin, RBC, and skin induration diameter were mitigated with the addition of feed additives.

CLINICAL RELEVANCE
The described model is a tool to evaluate the ability of feed additives to mitigate the immunosuppressive effects of Dex.

Keywords: dexamethasone, immune, keyhole limpet hemocyanin, sheep, feed additives
circulating glucocorticoid levels are elevated and can result in disease and metabolic disorders.\(^5\)

Dexamethasone (Dex) is a synthetic corticosteroid compound that binds to the same receptor as the naturally occurring hormone cortisol.\(^6\) Cortisol is linked to stress events due to activation of the hypothalamic-pituitary-adrenal axis, in turn, stimulating the release of cortisol from the adrenal cortex. Dex has been widely used as an immunosuppressant in ruminant animal research. For example, Guijarro et al.\(^7\) evaluated chronic Dex administration in sheep naturally affected by scrapie. They reported anti-inflammatory activity through the suppression of IL-6 and the IL-1 receptor in sheep brains. In a study\(^8\) with lambs treated with Dex every 48 hours, increased lymphocytes, monocytes, and leukocytes were observed compared to nontreated lambs. The effects of Dex have also been evaluated in beef steers, where chronic administration of Dex resulted in increased WBCs and neutrophils but decreased lymphocytes.\(^9\) Long-term administration of Dex in goats resulted in increased WBC counts, increased blood sugar levels, decreased dry matter intake, and decreased body weights (BWs) compared to controls.\(^10\) Wang et al.\(^11\) evaluated the ability of a commercial feed additive to mitigate the effects of Dex in sheep. They described that the mode of action of Dex is to suppress both the innate and adaptive immune response through the reduction in neutrophil L-selectin and IL-1β production in sheep.

Keyhole limpet hemocyanin (KLH) is a metalloprotein found in the great keyhole limpet (Megathura crenulata), a rare mollusk found off the western coast of the United States. Challenge with KLH has been used extensively in clinical trials over the past few decades. The ability of KLH to elicit an immune response has been comprehensively reviewed.\(^12,13\) The work described herein utilizes a long-term Dex treatment to mimic a stress event in conjunction with IM and ID challenge with KLH. The response was evaluated by looking at performance metrics as well as specific host responses to KLH. The overall hypothesis was that feed additives could mitigate the effects of Dex on host responses.

**Methods**

**Animals and housing**

All research procedures, animal care, and husbandry protocols were approved by the IACUC of the Purina Animal Nutrition Center. Hair-sheep rams less than 1 year of age (N = 60; BW, 28.6 ± 3.5 kg) were acquired from a local producer and acclimated to their new environment and feed for 21 days prior to the initiation of the 37-day study. Sheep were housed in individual pens (16 ft\(^2\)) within the Small Ruminant Facility of the Purina Animal Nutrition Center in Gray Summit, Missouri, for the duration of the study (from September 25, 2020, to November 2, 2020). Their diet consisted of ad libitum access to mixed-grass hay and water as well as a nutritionally complete, textured diet formulated for sheep offered at 3.5% BW that would be used as a carrier for feed additives.

**Treatment groups**

The rams were allocated to treatment groups based on average BW measured on 2 consecutive days immediately prior to starting the study. Treatment 1 (Trt 1) and Treatment 4 (Trt 4) groups were fed only the complete textured diet, without a feed additive. Treatment 2 (Trt 2) feed was top dressed with 18 g of a proprietary yeast and proprietary botanical extract blend feed additive each day. Treatment 3 (Trt 3) feed was top dressed with 4 g of a proprietary feed additive consisting of natural aluminosilicates and yeast components each day. Additive-fed groups (Trt 2 and Trt 3) were allocated 18 sheep each, whereas control-fed groups (Trt 1 and Trt 4) were allocated 12 sheep each.

**Feed additives**

The rams’ diet consisted of ad libitum access to mixed-grass hay and a commercially available textured sheep feed. Hay feeders were replenished daily, although hay intake was not measured. The complete textured sheep feed was limited fed at 3.5% of the average BW of each treatment group. Feed amount was adjusted following each BW measurement throughout the study (days –16, –7, 0, 7, 14, and 21). To properly administer the feed additive, the total amount of sheep feed needed for each treatment group was mixed with the total amount of feed additive needed in a small concrete mixer for 3 minutes. Due to the elevated molasses content of the sheep feed, the feed additive easily adhered to the feed. Limit feeding ensured that the sheep consumed all of their allotted treatment. There were no feed refusals.

**Immunosuppression and antigen inoculation**

Immunosuppressive treatments (Trt 2, Trt 3, and Trt 4) were injected with 0.1 mg/kg BW of Dex (Sparhawk Laboratories Inc). SC, twice daily (0800 and 1500 hours) from day –16 to day 21 of the study. The dosage amount was based on the average weight of all sheep in the study and was adjusted following each BW measurement. Sheep in the Trt 1 group were managed identically except that they did not receive Dex injections. A delayed-type hypersensitivity skin test was adapted from Hall et al.\(^14\) All treatment groups were immunized twice with KLH plus Freund incomplete adjuvant (FIA). On day 0 of the study, all treatment groups were given the initial immunization of KLH via a 500-μL injection of 500 μg KLH plus adjuvant, IM, into the neck region of the sheep. On day 14 of the study, a second 500-μL injection of 500 μg KLH with adjuvant, IM, was administered into the opposite side of the neck.

Also on day 14, the hair of the ventrolateral abdomen on each side of the sheep was shaved to a height of 1.5 mm using electric clippers in preparation for the ID challenge. The shaved portion of the abdomen was divided into 3 sections by marking 2 vertical lines 8 cm apart with a permanent marker. This created 3 separate locations for the ID injections, avoiding overlap of the subsequent reactions. On day 15 of
the study, all sheep were injected in the shaved portion of the abdomen with 0.05 mL of saline (0.9%), 0.05 mL of histamine (0.1 mg/mL; Histatrol; Center Laboratories), and 0.05 mL of heat-aggregated KLH, ID. Tuberculin syringes (1 mL) and 25-gauge needles were used to administer the ID injections.

Antigen preparation
The KLH-adjuvant immunization was prepared by mixing 2.0 mg/mL KLH (Milipore Sigma) diluted in 0.9% saline mixed with FIA (Thermo Scientific) for a final solution of 50% FIA. Heat-aggregated KLH was prepared by dissolving 120 mg of soluble KLH into 6 mL normal saline solution. The mixture was heat aggregated in an 80°C water bath for 1 hour. The resultant gel was then centrifuged twice at 400 X g for 10 minutes, removing the supernatant each time. The gel was then dispersed by passing it through a sterile 23-guage needle once and through a sterile 25-guage needle twice, carefully avoiding air bubbles. Heat-aggregated KLH was then dispersed at 0.05 mL into syringes for the ID injections.

Sample collection and measurements
Sample collection timepoints fell throughout the study (Table 1). BWs were measured on days −16, −7, 0, 7, 14, and 21. Blood was collected in a BD Vacutainer K2EDTA blood collection vacuum-tube via venipuncture prior to the daily Dex injection on days −16, 0, 7, 11, 14, and 21 of the study. Whole blood was analyzed for CBC parameters using a VETSCAN HM5 Hematology Analyzer (Zoetis). After ID injections were given on day 15, the diameter of the skin induration at each injection site was measured at set intervals (30, 180, 360, 540, 1,440, and 1,620 minutes postinjection) using a digital set of calipers.

Statistical analysis
A completely randomized repeated measures design was used to analyze the data. Analysis of variance was done with mixed models using the MIXED procedure in SAS (version 9.4; SAS Institute Inc), and least squares means were compared using the Fisher least significant difference test (P < .05) unless otherwise stated.

Results
Animal performance
Following 16 days of Dex injections, the mean BW among treatments began to diverge. The BW ± SD of Dex treated sheep (Trt 2, Trt 3, and Trt 4) was significantly lower (P < .001) on day 7 (27.95 ± 3.06, 28.67 ± 2.55, and 28.86 ± 3.49), day 14 (29.69 ± 2.75, 30.01 ± 2.68, and 30.22 ± 3.31), and day 21 (30.19 ± 2.86, 30.52 ± 2.72, and 30.47 ± 3.03) compared to no-Dex Trt 1 sheep at days 7, 14, and 21 (30.94 ± 2.75, 32.92 ± 2.50, and 32.89 ± 2.15). Similarly, a decrease was observed for average daily gain in Dex-treated sheep (P < .001; Figure 1), with the mean ± SD average daily gain across all data collection timepoints being significantly lower for Trt 2 (0.11 ± 0.06 kg), Trt 3 (0.12 ± 0.07 kg), and Trt 4 (0.14 ± 0.06 kg) compared to Trt 1 sheep (0.21 ± 0.07 kg; P < .001).

Complete blood count
The mean ± SD CBC parameters from each treatment group across all timepoints were evaluated (Table 2). Lymphocyte count, MCH, MCV, mean platelet volume, monocyte count, platelet hematocrit, total platelets, and WBC percentage
Table 2—Results of CBC analysis of sheep immunosuppressed with Dex and nonimmunosuppressed, with and without being fed a feed additive, performed using a VETSCAN HMS Hematology Analyzer (Zoetis).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Trt 1</th>
<th>Trt 2</th>
<th>Trt 3</th>
<th>Trt 4</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte count (g/dL)</td>
<td>3.463±a</td>
<td>5.222±b</td>
<td>5.455±b</td>
<td>5.393±b</td>
<td>0.0881</td>
<td>.001</td>
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<tr>
<td>Granulocyte (%)</td>
<td>27.25±a</td>
<td>39.07±b</td>
<td>40.30±b</td>
<td>40.12±b</td>
<td>2.2230</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.54±a</td>
<td>28.34±b</td>
<td>28.31±b</td>
<td>27.02±b</td>
<td>0.4827</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>119.26±a</td>
<td>102.21±b</td>
<td>101.15±a</td>
<td>95.69±b</td>
<td>2.2766</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>72.26±a</td>
<td>60.44±b</td>
<td>59.20±a</td>
<td>59.38±b</td>
<td>2.2229</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lymphocytes (10⁹/L)</td>
<td>9.1090a</td>
<td>7.9214a</td>
<td>7.8108a</td>
<td>7.5974a</td>
<td>0.0746</td>
<td>.184</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (g/dL)</td>
<td>367.01</td>
<td>359.74</td>
<td>356.14</td>
<td>354.06</td>
<td>0.0100</td>
<td>.067</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>10.7333</td>
<td>10.4451</td>
<td>10.6725</td>
<td>10.8361</td>
<td>0.0174</td>
<td>.395</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>29.21</td>
<td>29.09</td>
<td>29.99</td>
<td>30.68</td>
<td>0.5591</td>
<td>.252</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>5.2472</td>
<td>5.2611</td>
<td>5.2222</td>
<td>5.2819</td>
<td>0.0603</td>
<td>.884</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>11.14±a</td>
<td>9.84±a</td>
<td>9.49±a</td>
<td>8.96±c</td>
<td>0.2832</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Red blood cell count (10¹²/L)</td>
<td>438.71</td>
<td>467.25</td>
<td>429.44</td>
<td>429.68</td>
<td>23.3375</td>
<td>.484</td>
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<td>Platelet hematocrit (%)</td>
<td>0.0632</td>
<td>0.0661</td>
<td>0.0664</td>
<td>0.0646</td>
<td>0.0578</td>
<td>.901</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>0.2288</td>
<td>0.2448</td>
<td>0.2231</td>
<td>0.2264</td>
<td>0.0120</td>
<td>.429</td>
</tr>
<tr>
<td>White blood cells (%)</td>
<td>12.6354</td>
<td>13.2097</td>
<td>13.3332</td>
<td>13.0561</td>
<td>0.0581</td>
<td>.914</td>
</tr>
</tbody>
</table>

Mean CBC values for all timepoints are reported.

Means of CBC parameters for all sheep (N = 60) at all timepoints (days −16, 0, 7, 11, 14, and 21) are reported. Treatment groups consisted of not immunosuppressed and no feed additive (Treatment 1 [Trt 1]; n = 12), immunosuppressed and a yeast and botanical extract blend feed additive (Treatment 2 [Trt 2]; n = 18), immunosuppressed and a natural aluminosilicate and yeast component feed additive (Treatment 3 [Trt 3]; n = 18), and immunosuppressed with no feed additive (Treatment 4 [Trt 4]; n = 12).

Induration measurements

There were no measurable skin induration reactions to saline injections. The mean ± SE skin induration diameter reactions to Histatrol (mm) across all timepoints were largest in Trt 1 sheep (16.74 ± 1.11) followed by Trt 2 (12.75 ± 0.91), whereas Trt 3 and Trt 4 were the smallest and did not significantly differ (9.26, ± 0.91; 9.02, ± 1.11).

The mean ± SE skin induration diameter reactions to KLH (mm) across all measurement timepoints were significantly larger in the Trt 1 (23.08 ± 0.99) sheep compared to Trt 2, Trt 3, and Trt 4 sheep (12.39 ± 0.81, 13.17 ± 0.81, and 10.26 ± 0.99; P < .001; Figure 2). The evaluation of KLH skin induration diameter reactions over time (30 to 1,620 minutes postinjection) shows that the no-Dex Trt 1 reactions were largest from start to finish; the smallest reactions were found in Trt 4 animals, with Trt 2 and Trt 3 reactions lying between Trt 1 and Trt 4 (Figure 3).

Figure 2—Average keyhole limpet hemocyanin skin induration diameter (mm) by treatment over all data collection timepoints. Bars with differing superscripts are statistically significant (P < .001). The whiskers represent the SD within treatment group. Treatment groups consisted of nonimmunosuppressed and no feed additive (Treatment 1 [Trt 1]; n = 12), immunosuppressed and a yeast and botanical extract blend feed additive (Treatment 2 [Trt 2]; n = 18), immunosuppressed and a natural aluminosilicate and yeast component feed additive (Treatment 3 [Trt 3]; n = 18), and immunosuppressed with no feed additive (Treatment 4 [Trt 4]; n = 12).
In this study, we coupled the immunosuppressive effects of Dex with IM and ID challenge with KLH to evaluate the immune-modulating effects of feed additives in sheep. Daily injections with Dex resulted in significant differences in growth, CBC measurements, and response to ID challenge with KLH. The addition of feed additives mitigated the suppression of the immune response by Dex. However, the feed additives were unable to overcome the detrimental effects that Dex had on average daily gain.

Treatment with Dex has previously been reported to mitigate immune responses. For example, chronic Dex treatment in mallard ducks resulted in the suppression of antibody production, which was suggested to be caused by the suppression of phagocytic activity in lymphocytes. In a different study involving mallard ducks, Dex treatment resulted in a significant reduction in dermal reaction to phytohemagglutinin ID injection as well as a reduction in total antibody titers. In our study, the antibody response to KLH was evaluated; however, no significant difference between treatment groups was detected (data not shown). Previous studies have demonstrated that Dex has a dose-dependent effect on antibody response. Overall, this suggests that this study did not include a high enough dose of Dex to affect antibody response.

Previously, Hall et al described immune responses to KLH in healthy control sheep and sheep with clinical foot rot. They observed significantly larger induration diameter, resulting from ID challenge with KLH, in healthy sheep when compared to sheep with clinical foot rot. In the current study, challenge with KLH ID injection resulted in a larger induration diameter in non–Dex-treated sheep when compared to sheep that were immunosuppressed with Dex. One possible explanation is that both foot rot and challenge with Dex result in a similar mode of action to suppress the immune response. Further
research is necessary to understand the exact mechanism of these challenges on the immune system.

The inclusion of Trt 2 or Trt 3 feed additives was unable to mitigate the reduction in BW and average daily gain, but the lower BW gain in Trt 2, Trt 3, and Trt 4 animals confirms that the Dex injections did provide a challenge to the animals. The effects on intake and BW gain from corticosteroid treatment are likely dependent on dosage amount and duration of treatment. It has been previously reported that a singular 5-mg Dex trimethylacetate treatment and a 2-mg daily Dex sodium phosphate treatment for 10 days increased feed intake and weight gain in sheep entering pens and feed lots, and a weekly corticosteroid (1.33 mg/kg BW methylprednisolone acetate) injection mitigated the negative impacts on gain and intake caused by internal parasites. The contrasting results between these studies and the gain and intake caused by internal parasites. The acetate injection mitigated the negative impacts on corticosteroid (1.33 mg/kg BW during a 21-day Dex challenge using 0.2 mg/kg BW in goats. Repeated daily injections of Dex have also been shown to reduce BW in rats.

Significant differences were seen in several CBC parameters. This is not an unexpected result as previous research has also demonstrated changes in CBC. For example, in a study with lambs chronically treated with Dex, increased lymphocytes, monocytes, and leukocytes were observed compared to nontreated lambs. These results would indicate that Dex treatment caused a biological effect, but no conclusions can be drawn around the impact to the immune system.

A great benefit to this model is that it is pathogen free. The development of a pathogen-free challenge model in livestock is important for several reasons. First, pathogen-free models are a safer, more humane method for evaluating challenges to the animal’s immune system. Next, these models have no risk of contaminating or inadvertently transmitting an infectious agent. For these reasons, we set out to develop a pathogen-free model that could be used to evaluate the efficacy of feed additives on a challenge to the immune system in livestock animals. The development of this model will allow researchers to use a nonlethal, pathogen-free challenge to evaluate the effectiveness of feed additives quickly and efficiently for immune and performance-modulating properties.

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Disclosures

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References


