

Effects of Aerosolized Dust in Goats on Lung Clearance of *Pasteurella* and *Mannheimia* Species

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Abstract. The objective was to determine whether the inhalation of large quantities of feedyard dust predisposed the animals to pulmonary bacterial proliferation. Two control groups, C1 and C2, did not receive dust treatments, and two principal groups (P1 and P2) received a total of 14 dust treatments each. The C1 and P1 groups of goats each received a transthoracic challenge of live *Mannheimia haemolytica* (4×10^6 colony forming units, CFU). The C2 and P2 groups of goats each received a transthoracic challenge of live *Pasteurella multocida* (1.0×10^6 CFU/goat). The results showed that dusted animals had fever when compared with non-dusted controls. In addition, dusted animals demonstrated a leukocytosis with neutrophilia after the first dust treatment that was not sustainable. Finally, dusted animals demonstrated pulmonary clearance of two potential bacterial pathogens that was not significantly different from that shown by control (not dusted) animals.

Dust, in and around feedyards, is an industry problem [25]. Small dust particles (approximately 2.5 μm in diameter) are becoming increasingly scrutinized by regulatory agencies (e.g., the Environmental Protection Agency) because of their potential to harm human health [15, 23]. Indeed, many agricultural practices are currently being scrutinized for their potential to produce large amounts of fine dust particles [4, 13].

Feedyard dust is thought to affect the incidence of cattle pneumonia [12] and perhaps the growth potential of young calves. It is known that the inhalation of organic dust can be toxic to a number of animal species [22]. One of the most toxic components found in dust is endotoxin [19, 20], and endotoxin is produced by all Gram-negative bacteria. The outer membrane of these bacteria is composed of lipopolysaccharide molecules called endotoxin, and the most toxic part of the endo-

toxin molecule is the lipid A portion [11]. Feedyard dust should be expected to contain a high concentration of endotoxin because manure contains a high percentage of Gram-negative enteric bacteria (e.g., *Escherichia coli*), which are normal inhabitants of the large intestines of all animals. When manure dries and ruminant animals trample it into dust, it is then carried aloft by the movement of ruminants and with the prevailing winds. Culturable Gram-negative bacteria are frequently not found in the air because their cell membrane is very susceptible to desiccation [8, 14], but the resulting endotoxin-containing dust particles are very biologically active [2] and are free to enter the respiratory tract of feedyard animals [12].

Mannheimia haemolytica and *Pasteurella multocida* are involved with most acute fibrinohemorrhagic pneumonias that develop in market-stressed feeder/stocker calves, sheep, and goats after shipment [10]. This disease usually develops after ruminants are marketed and transported. During marketing stress, the ruminants often become exposed to many viral respiratory tract infections, especially herpes rhinotracheitis viral infections

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[9]. *M. haemolytica* is considered the primary bacterial pathogen in pasteurellosis that frequently develops in market-stressed cattle, sheep, and goats [1, 5, 6, 26]. The actual role of *P. multocida* as an agent in acute pneumonias of cattle, sheep, and goats is not well understood. However, it has been suggested that it should receive recognition as a primary pathogen in these animals [7].

Previous work has shown that goats are an excellent model for use in researching acute pneumonia [17, 18] and immunity [16]. The objectives of this study were to examine the ability of weanling principal goats dusted in a semi-air-tight tent compared with non-dust-treated weanling controls to clear live *M. haemolytica* or *P. multocida* injected transthoracically. The lungs of all goats were examined grossly and histologically, and the mean temperature response and the mean white blood cell response after dust treatment of the principal groups were compared with the responses of the non-dusted controls.

Materials and Methods

Bacteria. *Pasteurella multocida* A:3(PmA:3), was isolated from a confirmed fatal case of acute bovine respiratory disease [28]. *Mannheimia haemolytica* A1(MhA1) was also isolated from a confirmed case of acute bovine respiratory disease [24]. Cultures were stored at -70°C in reconstituted double-strength powdered milk on filter paper squares (0.5 cm). For use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar (Difco, Detroit, MI) plus 5% bovine erythrocytes. The frozen stock cultures were transferred only once before use in experiments. Colonies from the blood agar were used to initiate cultures on Brain Heart Infusion (BHI) agar plates. These cultures were washed with physiological saline and used as starter cultures. Live PmA:3 and MhA1 were obtained from an exponential phase culture (18 h) grown in 1 L of brain heart infusion broth in a 37°C shaker incubator set at 180 rpm. Cells were harvested by centrifugation (17,700g, 30 min, 4°C). The live cells were washed twice in sterile phosphate-buffered saline (PBS) and then adjusted to an optical density of 1.0 at 650 nm. The PmA:3 challenge dose was 1×10^6 CFU/ml in 1 ml of PBS, and the MhA1 challenge dose was 4×10^6 CFU/ml in 1 ml of PBS.

Goats. Twenty-four female weanling goats were housed in a three-sided barn. They were treated for internal helminth parasites (Ivomec MSD AGVET, Merck & Co., Inc., Rathway, NJ) and coccidia (Amprolium, MSD AGVET, Merck & Co., Inc.) Goats were limit fed a commercial pelletized ration (44% grain concentrate, 20% alfalfa hay, 30% cottonseed hulls and meal, 5% molasses, A and E vitamins, and trace minerals) and watered free choice. The goats were randomly allotted to control group 1 (not dusted but injected with PmA:3, $n = 6$), control group 2 (not dusted but injected with MhA1, $n = 6$), principal group 1 (dusted and injected with PmA:3, $n = 6$), and principal group 2 (dusted and injected with MhA1, $n = 6$). The goats were adjusted to their environment for 2 weeks before experimentation began, including, handling, feeding, and rectal temperature, and jugular blood sampling.

Tents. The semi-air-tight dust tent, 183 cm wide \times 244 cm long \times 213 cm tall (custom fabricated, Wolfe Canvas Co., Amarillo, TX) was made of 8 oz vinyl-coated polyester supported by a pipe frame. Zip-

pered doors at either end provided access and egress. The tent was divided into six stalls, each accommodating one or three goats, depending on the experiment. Prior to each 4-h dust treatment, the goats were randomized to the six pens inside the tent. Fifty-cm-diameter box fans were placed at each end inside the tent to cool and circulate the air. A 1.5-inch diameter PVC pipe ran from the outside, up the interior of the tent wall to a set of baffles, which was the outlet for the aerosolized dust located in the top center of the tent. A second tent of similar design was used to contain the non-dust control goats.

Feedyard dust preparation. Dried feed-yard manure was removed from a working feed yard and stored in plastic barrels. The dried manure was pulverized by passing it through 2-mm sieve roller drums (custom fabricated; Conservation and Production Research Lab, Bushland, Texas). The particles were then passed through a series of sieves (U.S. Standard Sieves; No. 100, opening 149 μm , and No. 60, opening 250 μm ; E.H. Sargent and Co., Chicago, IL), and the particles were further reduced in size by using an automated soil grinder (Nasco-Asplin, Fort Atkinson, WI). The dust (5.96% moisture) was stored in buckets with lids. Three 10-g samples of dust from each of three buckets were collected for endotoxin analysis. The samples were placed in specially prepared glass vials, vinyl capped, and sealed with aluminum seals. The glass vials were rinsed with reverse osmosis (RO) water (Millipore Reverse Osmosis Water System, 3216, Continental Water Systems, Lubbock, TX). The RO water and rinsed vials served as controls for background endotoxin.

Dust aerosol technique used to challenge the goats. The prepared feedyard dust (1500 g) was placed in a hopper (Accu Rate, Whitewater, WI) with a 1/4-inch auger in the bottom. The auger speed was set to deliver 990 g of dust over the 4-h dust treatment. The dust was augered into a metal funnel, which led to a Jet Mill (Jet-O-Mizer, Fluid Energy Processing and Equipments Co., Hatfield, PA) under 28 psi (air pressure regulators, Wilkerson, Grainger, Amarillo, TX) which further pulverized it and separated larger particles, which were lifted into a stainless steel trap (trap, custom fabricated, In-Tox Products, Albuquerque, NM). An air vibrator (operated with 10 psi of air) was attached to the outside of the hopper and touched the funnel, which prevented dust build-up in the funnel. The smaller particles from the jet mill were blown up a PVC pipe and out through PVC baffles located on the inside ceiling of the tent. Dust dissemination by the jet mill was augmented by air produced by a gasoline-powered portable air compressor (Air Compressor, Stewart-Warner, Johnson City, TN) and with air from a large blower motor (Portable Blower and Vacuum Motor, Industrial Type, 3hp, velocity linear fpm 33,570, Grainger, Amarillo, TX). The dust aerosol was collected for 30 min with three Andersen two-stage microbial cascade impactors and was analyzed for endotoxin concentration. The dust was trapped in two glass Petri dish bottoms, each containing 20 ml of sterile RO water. The Petri dishes were placed in the top stage-1 and the bottom stage-6 (hereafter called stage 0, and stage 00) (with stage-2 through stage-5 removed) of a six-stage Andersen microbial cascade impactor. (Note: only the pegs on the 6-stage Andersen impactor are small enough to accommodate the thick glass plates.) In addition, a series of three glass bottle traps, each containing 20 ml of RO water, were hooked into the impactor to trap the dust that might go through the impactor and be lost to the environment. After dust collection, the water from the three traps was combined into one sample. The samples analyzed for endotoxin concentration were the water from stage-1 (non-respirable particles), stage-6 (respirable particles), and the water from the traps (respirable particles). For the sake of reporting, stage-1 was called 0, and stage-6 was called 00. The Andersen impactors were placed 120 cm above the floor.

Endotoxin analysis. Endotoxin concentration in the dust was measured by using the kinetic chromogenic semi-quantitative Limulus amoebocyte lysate assay [29]. Aliquots of the extracts were serially diluted in 10-fold increments with pyrogen-free water. A 100- μ l aliquot of each dilution was mixed with 100 μ l of freshly prepared Limulus amoebocyte lysate containing chromogenic substrate in a pyrogen-free microliter plate that was kept at 37°C. Color development was monitored every 15 s with a microtiter plate reading spectrophotometer. The time interval required to reach 0.03 absorbance was compared with an endotoxin standard curve covering the range of 5 ng/ml to 0.5 pg/ml. The standards were linear over a 5-log range of a log-log plot. Endotoxin concentrations in unknown samples were calculated by linear interpolation from the standard curve. All dilutions were assayed in duplicate, and a parallel dilution was spiked with 50 pg of endotoxin to assess any enhancement or inhibition of activity by any of the extracted samples. Only those dilutions that did not exhibit enhancement or inhibition and which were parallel to the standard curve were used. Values were averaged on three different occasions. The coefficients of variation for these assays averaged 7.6%. To convert endotoxin activity to equivalent mass units of the EC-6 reference standards (U.S. Pharmacopeia), a factor of 10 EU/ng was used; EC-6 is a reference standard endotoxin, and EU signifies endotoxin units. The analysis of endotoxin was conducted at the IBT Reference Lab (Lenexa, KS).

Measurement of rectal body temperatures and complete blood cell counts (CBC) relative to dust treatment. The rectal temperatures and CBC counts were measured following a time series relative to dust treatment: pre-dust treatment, (4-h dust treatment), 4-, 8-, 12-, and 24-h post-dust treatment. The time series was modified for rectal temperatures and collection of blood for CBC counts when consecutive dust treatments were administered. The data were collected for rectal temperatures at 48 h, 24 h, and just before dust treatment administration and 4, 8, 12, and 24 h after the 1st dust treatment and 4 h after the 2nd dust treatment. Temperature measurements then lapsed to 24 h prior to bacterial challenge (BC), immediately prior to BC, and 24, 48, 72, and 96 h after BC. CBC counts were determined on the day prior to dust application and 10 and 14 days later.

Bacterial challenge, histologic examination and lung cultures. The goats were injected transhoracally with either 4×10^6 CFU/ml MhA1 or 1×10^6 CFU/ml PmA:3 immediately following the last dust treatment. Four days after the BC, the goats were taken to the Veterinary Medical Diagnostic Laboratory, Texas A&M University (Amarillo, Texas) for euthanasia and necropsy. The gross and histopathologic examinations of the tissues were performed blind. All lung lesions were observed, palpated, and measured. Specimens were obtained from all lung-injected sites for histologic examination and culturing for viable bacteria. Material for the bacterial isolates and their titers were obtained by first heat-sterilizing the lung surface of the area desired. A scalpel stab was made into the lung, and two sterile swabs were inserted into the site and allowed to absorb tissue fluid. One specimen was streaked on a blood agar plate, and the other specimen was washed and expressed into a tube containing 1.8 ml of physiologic saline solution. From this tube, a tenfold dilution series was made. One-tenth milliliter of each dilution was streaked onto blood agar plates in duplicate.

Injection of horse RBC. Horse RBC were collected in alsevers solution (anticoagulant) from the jugular vein of a horse. They were washed three times in sterile PBS and centrifuged at 50 rcf for 30 min at 4°C. One ml of horse RBC containing 1.7×10^9 cells in sterile PBS was injected into each goat intravenously after the first 4-h dust event. For the hemagglutination assay, the procedure of Carpenter was followed

[3]. This was done in order to examine the effect of dusting on the normal antibody response to equine RBCs.

Equipment used to quantify the dust and to determine the range of particle sizes. A five-stage cyclone device (In-tox Products, Albuquerque, NM) was used to quantify the small particles of dust <0.32 μ m that collected on the filter paper. Stage-five measured particles of 0.32 μ m; stage-four, particles of 0.65 μ m; stage three, particles of 1.4 μ m; stage-two, particles of 2.1 μ m; and stage-one, particles of 5.4 μ m. Eight Millipore Swinnex holders (Swinnex filter holders, Millipore Corp., Bedford, MA) (47 mm) equipped with a 0.45- μ m filter were used to quantitate the amount of dust in the air of the dust tent, by using Andersen vacuum pumps to collect 1 ft³ of air per min through the filters for 30 min. All determinations were done in duplicate. The filters were pre-weighed and after 30 min weighed again to determine the quantity of dust/m³ in the tent during the dust treatment. The duplicate samples were averaged. Five open Petri dishes were placed on the board that supported the cyclone device. These dishes were allowed to collect dust particles which settled out of the air during the 4-h period. These dishes were weighed before and after collection of the dust particles. A Malvern Mastersizer 2000 particle size analyzer (Malvern Instruments, Inc., Southborough, ME) with optical bench (dual 466-nm blue LED and ZmW63nm HeNe laser light source) was used to size and quantify the range of feed yard dust particles. This was done in triplicate.

Statistical analysis. The mean results for measured variables were compared by analysis of variance by using the General Linear Models procedures of SAS [21]. Means of rectal temperature, total WBC counts, absolute neutrophil and lymphocyte counts were compared between principal and control groups over the experiment, and within any sample day and period of time. Significant differences between treatment means were determined by Bonferroni's and Dunnett's adjusted paired *t*-test ($P \leq 0.05$), which allowed pairwise comparisons of treatment group means and control means within any sample collection day.

Results and Discussion

Analysis of dust. Feedyard dust contained mean 26.9 μ g of endotoxin per g according to the IBT Reference Laboratory analysis. The three histograms produced by the Malvern instrument were very similar to one another. The particle sizes ranged from 0.89 μ m to 355.6 μ m, with an average of 100.03 μ m. Two-stage Andersen impactors ($n = 3$) collected the following concentrations (ng) of endotoxin per ml over 30 min. Number one impactor, non-respirable plate 0, 97; respirable plate 00, 700; and combined traps, 25.1. Number two impactor, non-respirable 0, 65.9; respirable plate 00, 700; and combined traps, 45.6. Number three impactor, non-respirable plate 0, 35.1; respirable plate 00, 224; and combined traps, 9.22. Each unit contained 20 ml; therefore, each data point was multiplied by a factor of 20 for total concentration collected over 30 min. The mean endotoxin concentration from the non-respirable plate 0 was 1320 ng/20 ml (SEM 619); (respirable plate 00, plus combined traps) was 8293 ng (SEM 5406), over 30 min (pump 1 ft³/min). The following formula was used to

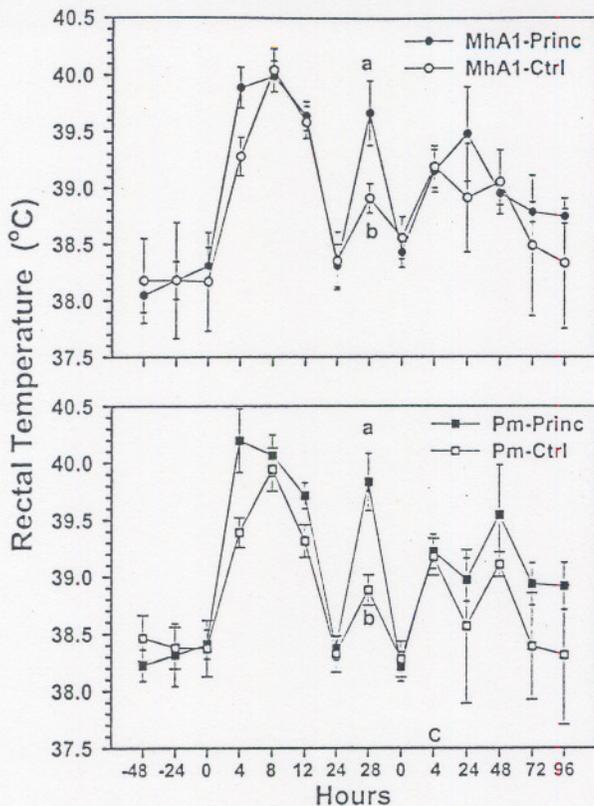


Fig. 1. Mean rectal temperature ($^{\circ}\text{C}$) of principal dust groups (both injected with PmA:3 or MhA1) and control (no dust) groups (both injected with PmA:3 or MhA1). The lower-case letters a and b indicate a significant rectal temperature increase in the principal dust group compared with the control (no dust) group. The error bars refer to the standard error of the means. A dust event occurred from 0 to 4 h, from 24 to 48 h, and from the second 0 to 4 h. The C above the second 4 h indicates that the goats were challenged with the appropriate bacteria immediately after that dust event. The principal group was treated with dust daily following the bacterial challenge.

determine the amount of endotoxin/ m^3 : $1 \text{ ft}^3 = 28.317 \text{ L} \times 30 \text{ min} = 849.51 \text{ L}$ divided by $1000 \text{ L} = 0.8495 \text{ m}^3$, which contained 8293 ng respirable endotoxin. Therefore, 1 m^3 of air contained 9672 ng of respirable endotoxin. Since one goat inspires 1.421 m^3 of air (in 4 h) $\times 9762 \text{ ng endotoxin/m}^3 = 13,872 \text{ ng}$ of respirable endotoxin was inspired over a 4-h dust treatment.

Analysis of feedyard dust during dust treatment. The hopper was charged with 1500 g of dust each time a 4-h dust treatment was given to the principal goats. The means for the following parameters were: weight of dust in the trap (464 g, SEM 38); weight of dust that entered the tent over 4 h (843 g, SEM 45); amount of dust in the tent ($89 \text{ g/m}^3/4 \text{ h}$, SEM 5); and the weight of dust determined for each stage of the cyclone device (small particles, 5 to $>1 \mu\text{m}$), stage 1 (0.343 g, SEM 0.042);

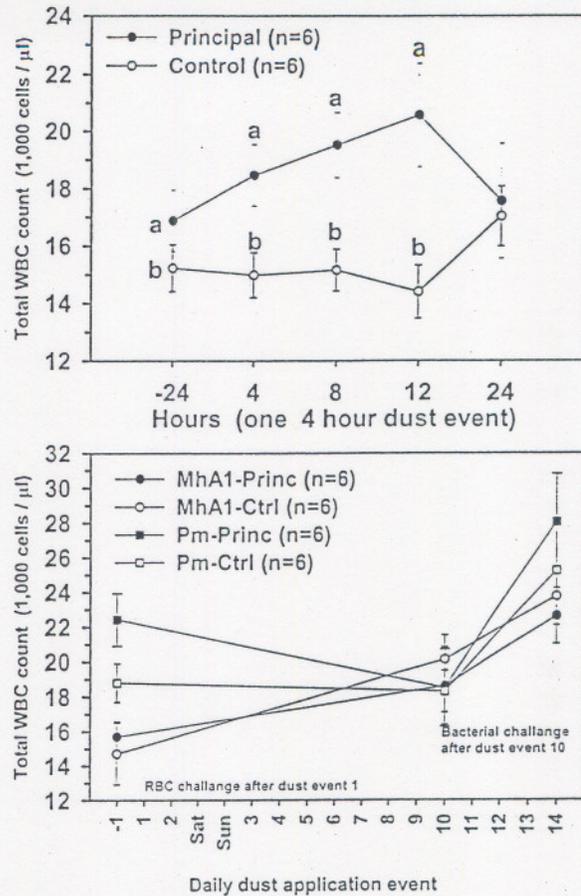


Fig. 2. Mean principal total white blood cell counts following dust treatments compared to non-dust treated controls. The top graph shows a typical principal group increase in mean leukocytes following one 4-h dust treatment. Significant differences between control and principal groups within day are indicated by a and b lower-case letters. After a 10 day pause, dust treatments were again started, the bottom graph shows the mean total WBC counts of the two principal and two control groups at three points, one day prior to dust application (-1), immediately prior to transthoracic injection of the respective bacterial challenges (10th dust treatment), and four days following bacterial challenge (14th dust treatment) immediately prior to necropsy. The error bars refer to the standard error of the means.

stage 2 (0.088 g, SEM 0.013); stage 3 (0.092 g, SEM 0.009); stage 4 (0.031 g, SEM 0.003); stage 5 (0.026 g, SEM 0.002); and the cyclone filter (0.008 g, SEM 0.0008); and weight of large dust particles passively settling into the five Petri dishes (1.002 g, SEM 0.590); weight of large dust particles passively settling over the tent (18.04 to 28.42 g/m^2).

The mean dust volumes collected from the tent by the Millipore filter technique were: 0 to 0.5 h, 49.628 mg/m^3 (SEM 4.92); 0.5 to 1.0 h, 46.869 mg/m^3 (SEM 3.90); 1.5 to 2.0 h, 47.877 mg/m^3 (SEM 7.43); 3.0 to 3.5 h, 47.217 mg/m^3 (SEM 1.91); and 3.5 to 4.0 h,

Table 1. Number of MhA1 and PmA:3 isolates recovered at necropsy from various anatomical locations, and mean titer at the challenge exposure site in the right lung

Groups	Nasal mucosa	Pharyngeal mucosa	Trachea		Thoracic cavity	Right lung	Mean \pm SD(cfu/ml) ^a	Liver	Spleen	Kidney
			Proximal part	Distal Part						
Control plus MhA1 (<i>n</i> = 6)	0/6 ^b	1/6	4/6	2/6	0/6	0/6	—	0/6	0/6	0/6
Dusted plus MhA1 (<i>n</i> = 6)	0/6	2/6	4/6	3/6	0/6	0/6	—	0/6	0/6	0/6
Control plus PmA:3	0/6	1/6	5/6	2/6	1/6	2/6	$6.28 \times 10^2 \pm 3.72 \times 10^2$	1/6	1/6	0/6
Dusted plus PmA:3	0/6	1/6	3/6	1/6	0/6	1/6	$8.0 \times 10^1 \pm 0.0$	0/6	0/6	0/6

^a MhA1 and PmA:3 titer calculated from 0.1 ml of tissue fluid taken from the right lung challenge site at necropsy; there were no significant differences in titer among the four groups; colony forming unit (CFU) data are expressed as mean \pm SD.

^b The numerator is the number of goats from which bacterial isolates were recovered; the denominator is the total number of goats.

70.755 mg/m³ (SEM 10.16). The grand mean was 50.591 mg/m³ (SEM 10.74).

Clinical effects of dusting. No panic or restlessness was observed among the principal goats during the 4-h dust treatment, although occasionally some goats would cough. Coughing in general seemed to increase for 1–2 h after the principals were returned to their pens. When the principal goats were removed from the dust tent, their hair coat was very dark with dust particulate matter. The ambient temperature was extremely hot (37.8°C) during the first dust treatment, and the controls and principals confined in different tents had similar mean temperatures (Fig. 1). The principal group's (*n* = 12) mean rectal temperatures significantly increased at 4 h ($P \leq 0.01$) post-dust treatment on the second day only, compared with the controls. A state of tolerance appeared to develop after repeated dust treatments (Fig 1) [20].

The mean total CBC counts for the dusted and non-dusted groups indicate that there was a significant difference ($P \leq 0.05$) in mean total WBC counts between the control and the principal group after one dust treatment (Fig. 2). Dusting of animals appeared to have no effect on their ability to respond to a particulate antigen (horse RBC) regarding antibody production (data not shown).

Gross and microscopic pulmonary lesions and *Pasteurella* and *Mannheimia* isolations. Among the control goats (*n* = 12), one goat exhibited a generalized bronchopneumonia, and one goat exhibited a severe exudative fibrinocellular pleuritis. Three of the 12 control goats had segmented pleural edema. No significant lesions were observed in four of the goats. In the principal goats, the only significant observed lesion was a severe exudative pneumonia in the appropriately challenged animals of all four groups. No cross-infections occurred between specific

challenged groups. However, both control and dusted animals effectively cleared MhA1 from their lungs, while two of the control goats and one of the dusted goats still had viable PmA:3 in their lungs (Table 1).

Few animal studies (of any kind) have attempted to determine the effects of the inhalation of endotoxin-laden dust. The few aerosol studies failed to show a negative endotoxin effect. A study examining pigs used flour dust (1–15 mg/m³) and dust-borne endotoxin (50–2500 ng/m³) exposure for 6 days [27]. These workers reported no endotoxin effects in the pigs. This may have been related to time of sample collection rather than the inability of the pigs to respond in a timely manner. The endotoxin in the feedyard dust treatment in our study caused the goat rectal temperatures to increase significantly above that of the control groups 4 h after the 2nd endotoxin/dust exposure (Fig. 1), and a dramatic leukocytosis occurred over 4–12 h after the first dust event (Fig. 2). Recent research confirmed that it is the endotoxin part of the feedyard dust that causes the biological activity, and not the microbes [19]. It was noted that the stress of several dust treatments caused a temporary decrease in their appetite for 4 days compared with control non-dusted goats; this was determined by weighing feed and again weighing any feed refused 24 h later (Purdy CW et al., submitted). It appears that on repeated dust treatments, rectal temperature tolerance occurs in goats. This state of tolerance was also observed in sheep treated with multiple endotoxin/dust treatments, by determining their rectal temperature and total WBC counts [20]. Alveolar macrophages were determined to have gray, dust-like particles that are difficult to see; however, when carbon black particles in sheep were inhaled (similar in size to the feedyard dust particles), it was easy to dust had little effect on the ability of goats to clear either large concentrations of MhA1 (4×10^6 CFU) or PmA:3

(1×10^6 CFU) injected into the right lung, with one exception in the principal PmA:3 group. Dusted goats cleared both these organisms as well as did control or non-dusted goats, with two exceptions in the control PmA:3 group (Table 1). Therefore, it appears that even though alveolar macrophages ingest dust particles, this does not appear to affect their ability to ingest and kill potential bacterial pathogens in the majority of goats. Inhalation of feedyard dust does not appear to affect the clearance of MhA1; however, one principal PmA:3-challenged goat did not clear the bacterial challenge 4 days later. Also, two non-dust-treated control PmA:3 goats did not clear the bacteria 4 days post challenge.

It is clear from this study that the inhalation of large quantities of endotoxin-laden feedyard dust did not predispose the animals in this experiment to pulmonary bacterial proliferation. This was unexpected. It was anticipated that dust-filled lung macrophages exposed to endotoxin would not be efficient in removing the bacterial challenge.

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