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SUSCEPTIBILITY OF PHAGOCYTES FROM ELK, DEER, BIGHORN SHEEP, AND DOMESTIC SHEEP TO *PASTEURELLA HAEMOLYTICA* CYTOTOXINS

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ABSTRACT: Alveolar macrophages and peripheral blood neutrophils from elk (*Cervus elaphus*), bighorn sheep (*Ovis canadensis canadensis*), and domestic sheep were exposed to culture supernatants from *Pasteurella haemolytica* isolated from bighorn sheep and domestic sheep. In a second experiment, peripheral blood neutrophils from mule deer (*Odocoileus hemionus*), elk, and bighorn sheep were exposed to culture supernatants from *P. haemolytica* isolated from elk, bighorn sheep and domestic sheep. Alveolar macrophages from elk, bighorn sheep and domestic sheep were resistant to killing by *P. haemolytica* supernatants from bighorn sheep and domestic sheep; susceptibility of neutrophils to cell death, as measured by release of lactate dehydrogenase, differed significantly ($P < 0.05$) between the four species tested. Bighorn sheep and domestic sheep neutrophils were susceptible to cytotoxin damage by the *P. haemolytica* isolates used; bighorn sheep neutrophils were four- to eight-fold more susceptible to cytotoxin damage than domestic sheep neutrophils. Neutrophils from deer and elk were resistant to killing by *P. haemolytica* cytotoxins from any species tested.

Key words: *Pasteurella haemolytica*, cytotoxin, neutrophil, alveolar macrophage, bighorn sheep, deer, elk, domestic sheep.

INTRODUCTION

Respiratory disease caused by pasteurellosis results in mortality in both bighorn sheep (*Ovis canadensis canadensis*) and domestic sheep species, yet it is reported less frequently in deer (*Odocoileus* spp.) or elk (*Cervus elaphus*) (Thorne et al., 1982; Franson and Smith, 1988). *Pasteurella haemolytica* can be isolated from both domestic sheep (Frank, 1982) and bighorn sheep (Wild and Miller, 1991) whether respiratory disease symptoms exist or not. Bighorn sheep apparently are more susceptible to respiratory infections than domestic sheep (Foreyt, 1988) and this apparent difference can be partially explained by the greater sensitivity of bighorn sheep neutrophils to cytotoxin-dependent killing by *P. haemolytica* organisms (Silflow et al., 1993).

Contact transmission of *P. haemolytica* from domestic sheep to bighorn sheep populations may have devastating consequences to bighorn sheep survival. The observation of mortality losses in free ranging bighorn sheep exposed to domestic sheep is confirmed by the results of experimental trials in which transmission of *P. haemolytica* between these two sheep species were tested on captive bighorn sheep (Foreyt, 1988; Onderka and Wishart, 1988). Consistently, there was high mortality in bighorn sheep but not in domestic sheep. Important questions extending from these results include whether other wild species, such as deer and elk, also are susceptible to mortality from *P. haemolytica*, and whether deer or elk may serve as a reservoir of pathogenic *P. haemolytica* which can be transmitted to bighorn sheep. Previous experiments involving deer and elk contact with bighorn sheep did not result in observed respiratory disease in any of the animals (Foreyt, 1992).

The cytotoxin produced by *P. haemolytica* isolates is an important virulence factor in the development of respiratory disease in many ruminant species (Nicolet, 1990). Colonization of the lower respiratory tract with *P. haemolytica* results in exogenous release of a soluble toxin capable of exacerbating the acute inflammation which is characteristic of pasteurellosis (Baluyut et al., 1981). Previous

workers have focused primarily on the neutrophil as the target phagocytic cell susceptible to cytotoxin mediation (Confer et al., 1990; Silflow et al., 1993). Czuprynski et al. (1991) found that at both low and high concentrations, the cytotoxin isolated from cattle causes activation or lysis of the neutrophil resulting in the release of intracellular components which can cause damage to the integrity of the lung. In addition to the neutrophil, cytotoxin mediation occurs in other immune cells, including suppressed proliferation of both bovine peripheral mononuclear cells (Czuprynski and Ortiz-Carranza, 1992) and lymphocytes (Majury and Shewen, 1991a, b), and has both lethal and sublethal effects on alveolar macrophages from cattle (Markham and Wilkie, 1980; Markham et al., 1982) and sheep (Sutherland et al., 1983).

Alveolar macrophages are the phagocytic cells responsible for initial defense of the lung against any infectious or non-infectious agent entering the lower airways (Liggitt, 1985). Silflow et al. (1989) found no apparent differences between bighorn and domestic sheep in the numbers of phagocytic cells in the alveolar spaces, nor in the phagocytic or bactericidal activities of alveolar macrophages. During early exposure of *P. haemolytica* to the lower respiratory tract, the first phagocytic cells to encounter cytotoxin are alveolar macrophages.

Our objectives were to determine whether alveolar macrophages from bighorn sheep, domestic sheep, and elk were susceptible to toxicity by *P. haemolytica* supernatants from bighorn sheep or domestic sheep, and to test for differences in bighorn sheep, domestic sheep, deer and elk neutrophil sensitivity to cytotoxin from isolates from bighorn sheep, domestic sheep, and elk.

MATERIALS AND METHODS

Three Rocky Mountain bighorn sheep, three domestic sheep, and three elk were used as sources of both neutrophils and alveolar macrophages for Experiment 1 in this study conducted between 19 January and 19 May 1993. The bighorn sheep (two females, one male) were 1-yr-old and the elk (three males) ranged in age from 1 to 2 yr. In Experiment 2, conducted between 1 March and 7 April 1993, five 1-yr-old Rocky Mountain bighorn sheep (two females, three males), two 1-yr-old female mule deer (*Odocoileus hemionus*), and three male 1- to 2-yr-old elk were used as a source of neutrophils. The bighorn sheep, deer and the elk from both experiments were from a captive herd (Washington State University, Pullman, Washington, USA). Three conventionally-reared female domestic sheep ranged in age from 3 to 4 yr. All animals were clinically healthy when samples were collected. However, in Experiment 1, we noted that one of the elk samples contained 50% neutrophils plus an unusually high number of eosinophils (47%) which were inseparable from the neutrophils. The results for this animal were excluded from the neutrophil sensitivity assay since these mixed-population cells were significantly ($P < 0.05$; least squared differences Statistix version 3.5, Analytical Software, St. Paul, Minnesota, USA) less susceptible to cytotoxin killing than cells from the remaining two elk which consisted of >70% and 90% neutrophils, respectively.

Animals were sedated intramuscularly using 0.5 mg/kg of xylazine hydrochloride (Rompun, Miles Laboratories, Bayvet Division, Shawnee, Kansas, USA). Alveolar macrophages were obtained for study using the lavage method of Silflow and Foreyt (1988). A veterinary laryngoscope was used to aid insertion of a section of plastic tubing 145 cm long and 6 mm in outside diameter into the trachea. After gently lodging the plastic tubing in a distal bronchus, 60 ml aliquots of sterile physiological saline were infused and immediately withdrawn. A total of 300 ml of fluid were instilled and withdrawn without any deleterious effects. The sedative effect of the xylazine was reversed by intravenous administration of 10 mg of yohimbine hydrochloride (Antagonil, Wildlife Laboratories Inc., Fort Collins, Colorado, USA). The recovered lavage fluid was centrifuged at 350 × *G* and the cell pellet resuspended in Hanks Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, New York, USA) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA). The alveolar cell population was characterized by staining a cytocentrifuge-prepared slide with Dif-Quik Kit (American Scientific Products, McGaw Park, Illinois, USA). Cells were counted using a hemocytometer (American Optical Corporation, Buffalo, New York), and cell viability was determined by trypan blue exclusion (Boyse et al., 1964). Typical yields were >90% macrophages, and these cells

exhibited >90% viability. For each experiment, cells were adjusted to a concentration of 2×10^6 cells/ml in HBSS + 1% FBS.

Peripheral blood samples were collected by jugular venipuncture into citrate phosphate dextrose solution (Sigma Chemical Company, St. Louis, Missouri, USA). Following centrifugation at 850 × *G* for 15 min, the plasma and buffy coats were discarded. Hypotonic lysis of red cells was accomplished by the addition of 45 ml distilled water for 45 sec followed by the addition of 5 ml of 10× phosphate buffered saline. Following centrifugation at 600 × *G* for 10 min, the lysis and centrifugation steps were repeated, and the final cell pellets were resuspended in HBSS + 1% FBS. Cells were counted using a hemocytometer, and cell viability was determined by trypan blue exclusion. Typical yields were >90% neutrophils, and these cells exhibited >90% viability. For each experiment, cells from each animal were adjusted to a concentration of 5×10^6 cells/ml in HBSS + 1% FBS.

In Experiment 1, isolates of *P. haemolytica* from two pneumonic bighorn sheep (T3,4,10 from the spleen; A2 from the lung) and one healthy domestic sheep (A2 from the pharynx) were used. These particular isolates were selected to represent both high and low levels of expected cytotoxin production. In Experiment 2, isolates from one healthy domestic sheep (A2 from the pharynx), two healthy bighorn sheep (T3,4 and T3,4,10 from the pharynx), and two healthy elk (T3,4 and T3,4,10 from the pharynx) were used. The A2 isolate was chosen for this experiment as a positive control because of its high level of cytotoxin production. Cytotoxins were isolated from culture supernatants using the method of Shewen and Wilkie (1982). Individual *P. haemolytica* isolates were cultured onto 5% sheep blood agar plates (Beckton Dickinson Microbiological Systems, Cockeysville, Maryland, USA) and incubated for 18 hr at 37 C. As a negative control, a different Gram-negative bacterium, *Enterobacter cloacae* (ATCC #35030), was handled identically. Several morphologically similar colonies were used to inoculate 100 ml of brain-heart infusion broth (Difco Laboratories, Detroit, Michigan, USA) which was incubated at 37 C until cultures reached early logarithmic growth. To count the number of bacteria, the optical densities of the cultures were measured at a wavelength of 600 nm (OD_{600}) until 1 OD_{600} (8×10^8 bacteria/ml) was reached (Maniatis et al., 1982). Bacteria were centrifuged for 10 min at 6,000 × *G* to a pellet, and resuspended in 30 ml of RPMI-1640 media (Gibco Laboratories) containing 7% FBS. Following incubation for 1 hr at 37 C, the bacteria again were centrifuged at 6,000 × *G* for 10 min, and the culture supernatants were removed and filter sterilized by passage through a 0.45 μm filter (Sigma Chemical Company). Culture supernatants were dialyzed (6,000 to 8,000 molecular weight cutoff) (Spectrum Medical Industries, Los Angeles, California, USA) to exhaustion against distilled water, at 4 C, and lyophilized.

We characterized the relative potency of toxins produced by various *P. haemolytica* isolates by adding bacterial culture supernatants to alveolar macrophages and neutrophils in vitro. Neutrophils from every animal were tested with every supernatant. Cytotoxicity was determined by assessing the release of lactate dehydrogenase (LDH) into the culture medium (Korzeniewski and Callewaert, 1983). Cytotoxicity was determined at final concentrations of supernatant of 150, 100, 50, 5, 0.5, and 0.05 μg/50 μl. All of the samples were resuspended in HBSS containing 1% FBS prior to the assay. Fifty μl of each supernatant preparation containing cytotoxin were added to the wells of 96-well plates, followed by the addition of either 1×10^5 macrophages or 2.5×10^5 neutrophils in 50 μl of HBSS containing 1% FBS to each well. Following 1 hr incubation at 37 C, 100 μl of LDH substrate were added. Levels of the reduced LDH substrate were determined on a Titertek 96-well plate reader (Flow Laboratories, McLean, Virginia, USA) coupled to an on-line IBM-XT computer (International Business Machines, Boca Raton, Florida, USA). All samples were compared to either macrophages or neutrophils treated with a 0.5% solution of the detergent saponin (Sigma Chemical Company) (maximal release) and untreated cells (background release) and the results recorded as a percentage of LDH released from untreated cells. The potency of the various cytotoxins was determined by comparing the 50% effective dose (ED_{50}). The ED_{50} is defined as the concentration of supernatant (μg/50 μl) which resulted in the death of 50% of the cells as determined by a graphic plot constructed to determine the intersection of 50% cell death and supernatant concentration.

Data were tested for statistical significance using the statistical software package Statistix version 3.5 (Analytical Software, St. Paul, Minnesota, USA) by first performing analysis of variance (ANOVA), then testing for differences between means using least squared differences (LSD) ($P < 0.05$).

RESULTS

In Experiment 1, alveolar macrophages from all species tested were not susceptible to killing by any of the *P. haemolytica* supernatants used (Table 1). Furthermore,

TABLE 1. Effects of bacterial culture supernatants tested for cytotoxicity against alveolar macrophages and neutrophils from bighorn sheep, domestic sheep and elk in experiment 1.

Culture supernatant	*Enterobacter* sp. or *Pasteurella haemolytica* biotype or serotype	Alveolar macrophage source			Neutrophil source		
		Bighorn sheep	Domestic sheep	Elk	Bighorn sheep	Domestic sheep	Elk
1	*Enterobacter cloacae*	>150[a]	>150	>150	>150	>150	>150
2	A2[b]	>150	>150	>150	12	48	>150
3	T3,4,10[c]	>150	>150	>150	>150	>150	>150
4	A2[c]	>150	>150	>150	8	68	>150

[a] 50% effective dose (ED_{50}) = the concentration of supernatant (μg/50 μl) which results in death of 50% of the cells as determined by a graphic plot constructed to determine the intersection of 50% cell death and supernatant concentration.
[b] Isolate from a healthy domestic sheep.
[c] Isolate from a pneumonic bighorn sheep.

neutrophils from elk also were resistant to cytotoxin lysis at concentrations as high as 150 μg/50 μl (highest concentration tested). Supernatants from the negative control *Enterobacter* and from one *P. haemolytica* isolate (T3,4,10 from the spleen of a pneumonic bighorn sheep) were non-cytotoxic at all doses tested against both macrophages and neutrophils from bighorn sheep, domestic sheep, and elk. Two *P. haemolytica* supernatants, one from a healthy domestic sheep isolate (A2 from the pharynx) and one from a pneumonic bighorn sheep isolate (A2 from the lung), were cytotoxic for neutrophils from both bighorn sheep and domestic sheep (Table 1). The relative cytotoxic potency of these supernatants was significantly greater (4- to 8-fold, $P < 0.05$) against bighorn sheep neutrophils than domestic sheep neutrophils.

In Experiment 2, neutrophils from deer and elk were resistant to lysis by culture supernatants from all *P. haemolytica* isolates at concentrations as high as 150 μg/50 μl (Table 2). But bighorn sheep neutrophils were susceptible to lysis by two isolates, one from a healthy bighorn sheep (T3,4,10 from the pharynx) and one from a healthy domestic sheep (A2 from the pharynx). Supernatants from the negative control *Enterobacter*, one isolate from a healthy bighorn sheep (T3,4 from the pharynx), and two isolates from healthy elk (T3,4 and T3,4,10 both from the pharynx) were non-cytotoxic to neutrophils from bighorn sheep, deer and elk.

DISCUSSION

Based on our results, we believe that the neutrophil, rather than the alveolar macrophage, is the primary phagocytic cell susceptible to lysis by the cytotoxin preparations of *P. haemolytica* isolates. Neither elk, domestic sheep nor bighorn sheep alveolar macrophages were susceptible to lysis by the cytotoxins used in this study. Neutrophils from deer and elk were not susceptible to cytotoxin-mediated cell death, but lysis did occur in neutrophils from bighorn sheep and domestic sheep. The in vitro range of neutrophil susceptibility to cell death may reflect the observation that pneumonic pasteurellosis is more prevalent in bighorn sheep compared to domestic sheep, and is seldom documented in deer or elk.

The mechanism for cytotoxin cell lysis involves the formation of ion channels in the cell membrane that increase membrane permeability and cause biological effects ranging from cell activation to cell death (Clinkenbeard et al., 1989). The observation that neutrophils are more susceptible to cytotoxin lysis than macrophages is evidence that the neutrophil lipid membrane is less stable than that of the macrophage; alternatively, different receptor levels may exist on neutrophils compared with macrophages. Recognition of

cytotoxin by cell receptors has not been definitively demonstrated, but the toxic effects of cytotoxin was interrupted in some cases when beta-adrenoceptor antagonists were used (Henricks et al., 1990). The glycolipid, GM 1, is the cell receptor for cholera toxin (Holmgren et al., 1973) and cholera toxin's B subunit works through a similar mechanism as *P. haemolytica* cytotoxin to increase intracellular calcium influx. Majury and Shewen (1991b) propose that the *P. haemolytica* cytotoxin, like cholera toxin, may affect calcium flux via a glycolipid on the cell surface or via a second cell surface component associated with the glycolipid. Possible differing receptor densities between species and between cell types may explain the observed differences in cytotoxin susceptibility. Furthermore, we have observed that concentrations of supernatants greater than 150 μg/50 μl result in lower neutrophil killing percentages than concentrations at or near 150 μg/50 μl. Because the supernatant preparations contain many proteins present in fetal bovine serum in addition to the cytotoxin protein, these proteins actually may block access of the cytotoxin to the cell under the in vitro conditions of the assay.

The absence of alveolar macrophage cell death should not be interpreted to mean that no role is played by the macrophage in the pathogenesis of respiratory disease in bighorn sheep. Indeed, sublethal concentrations of cytotoxin and endotoxin regulate the phagocytic function and the production of soluble factors by alveolar macrophages (Markham et al., 1982). In bovine alveolar macrophages, phagocytosis and chemotactic factor release are impaired by cytotoxin, while *P. haemolytica* type 1 endotoxin stimulates alveolar macrophage release of TNF-alpha (Bienhoff et al., 1992). These soluble factors play important roles in lung defense by recruiting neutrophils into the lung for protection against microorganisms, yet the same protective features of neutrophil respiratory burst and degranulation can contribute to local tissue injury (Czuprynski et al., 1991). Protection of bighorn sheep populations from the devastating effects of pasteurellosis depends on understanding the mechanisms involved in the disease process. The production of cytotoxin is one of several possible virulence factors involved in the development of pasteurellosis, and based on our observations, we propose that it may be one of the most important mechanisms for the cause of respiratory disease in bighorn and domestic sheep. Use of the cytotoxin assay to determine differential potencies of various *P. haemolytica* isolates has proven useful in identifying whether isolates obtained from individual animals or herds should be considered potentially lethal. Furthermore, the cytotoxicity assay has high correlation with neutrophil sensitivity in animals highly susceptible to pasteurellosis (bighorn sheep) and animals relatively resistant to pasteurellosis (deer and elk). Future research efforts focusing on the prevention of pasteurellosis should emphasize the alteration or attenuation of the cytotoxin-mediated effects on neutrophils.

TABLE 2. Effects of bacterial culture supernatants tested for cytotoxicity against neutrophils from bighorn sheep, deer and elk in experiment 2.

Culture supernatant	*Enterobacter* or *Pasteurella haemolytica* biotype/serotype	Neutrophil source		
		Bighorn	Deer	Elk
1	*Enterobacter cloacae*	>150[a]	>150	>150
2	A2[b]	12	>150	>150
3	T3,4,10[c]	125	>150	>150
4	T3,4[c]	>150	>150	>150
5	T3,4,10[d]	>150	>150	>150
6	T3,4[d]	>150	>150	>150

[a] 50% effective dose (ED_{50}) = the concentration of supernatant (μg/50 μl) which results in death of 50% of the cells as determined by a graphic plot constructed to determine the intersection of 50% cell death and supernatant concentration.
[b] Isolate from a healthy domestic sheep.
[c] Isolate from a healthy bighorn sheep.
[d] Isolate from a healthy elk.

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LITERATURE CITED

BALUYUT, C. S., R. R. SIMONSON, W. J. BEMRICK, AND S. K. MAHESWARAN. 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: Identification and partial characterization of a cytotoxin. American Journal of Veterinary Research 42: 1920–1926.

BIENHOFF, S. E., G. K. ALLEN, AND J. N. BERG. 1992. Release of tumor necrosis factor-alpha from bovine alveolar macrophages stimulated with bovine respiratory viruses and bacterial endotoxins. Veterinary Immunology Immunopathology 30: 341–357.

BOYSE, E. A., L. J. OLD, AND I. CHOURLINKOV. 1964. Cytotoxic test for demonstration of mouse antibody. Methods in Medical Research 10: 39–47.

CLINKENBEARD, K. D., D. A. MOSIER, AND A. W. CONFER. 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. Infection and Immunity 57: 420–425.

CONFER, A. W., K. R. SIMONS, M. T. BARRIE, AND K. D. CLINKENBEARD. 1990. Effects of *Pasteurella haemolytica* leukotoxin on neutrophils from white-tailed deer and several exotic ruminant species. Veterinary Research Communications 14: 175–180.

CZUPRYNSKI, C. J., AND O. ORTIZ-CARRANZA. 1992. *Pasteurella haemolytica* leukotoxin inhibits mitogen-induced bovine peripheral blood mononuclear cell proliferation *in vitro*. Microbial Pathogenesis 12: 459–463.

———, E. J. NOEL, O. ORTIZ-CARRANZA, AND S. SRIKUMARAN. 1991. Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. Infection and Immunity 59: 3126–3133.

FOREYT, W. J. 1988. Fatal *Pasteurella haemolytica* pneumonia in bighorn sheep following direct contact with normal domestic sheep—An experimental study. American Journal of Veterinary Research 50: 341–344.

———. 1992. Experimental contact association between bighorn sheep, elk, and deer with known *Pasteurella haemolytica* infections. Biennial Symposium of the Northern Wild Sheep and Goat Council 8: 213–218.

FRANK, G. H. 1982. Serotypes of *Pasteurella haemolytica* in sheep in the midwestern United States. American Journal of Veterinary Research 43: 2035–2037.

FRANSON, J. C., AND B. L. SMITH. 1988. Septicemic pasteurellosis in elk (*Cervus elaphus*) on the United States National Elk Refuge, Wyoming. Journal of Wildlife Diseases 24: 715–717.

HENRICKS, P. A. J., G. J. BINKHORST, A. A. DRIJVER, H. VAN DER VLIET, AND F. P. NIJKAMP. 1990. The effect of *Pasteurella haemolytica* cytotoxin on bovine polymorphonuclear leukocytes can be attenuated by beta-adrenoceptor antagonists. Veterinary Microbiology 22: 259–266.

HOLMGREN, J., I. LONNROTH, AND L. SVENNERHOLM. 1973. Tissue receptor for cholera exotoxin: Postulated structure from studies with GMI ganglioside and related glycolipids. Infection and Immunity 8: 208–214.

KORZENIEWSKI, C., AND D. M. CALLEWAERT. 1983. An enzyme-release assay for natural cytotoxicity. Journal of Immunological Methods 64: 313–320.

LIGGITT, H. D. 1985. Defense mechanisms in the bovine lung. Veterinary Clinics of North America: Food Animal Practice 1: 347–366.

MAJURY, A. L., AND P. E. SHEWEN. 1991a. The effect of *Pasteurella haemolytica* A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. Veterinary Immunology and Immunopathology 29: 41–56.

———, AND ———. 1991b. Preliminary investigation of the mechanism of inhibition of bovine lymphocyte proliferation by *Pasteurella haemolytica* A1 leukotoxin. Veterinary Immunology and Immunopathology 29: 57–68.

MANIATIS, T., E. F. FRITSCH, AND J. SAMBROOK. 1982. Propagation and maintenance of bacterial strains and viruses. *In* Molecular cloning: A laboratory manual, T. Maniatis, E. F. Fritsch, and J. Sambrook (eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p. 61.

MARKHAM, R. J., AND B. N. WILKIE. 1980. Interaction between *Pasteurella haemolytica* and bovine alveolar macrophages: Cytotoxic effect on macrophages and impaired phagocytosis. American Journal of Veterinary Research 41: 18–22.

———, M. L. RAMNARAINE, AND C. C. MUSCOPLAT. 1982. Cytotoxic effect of *Pasteurella haemolytica* on bovine polymorphonuclear leukocytes and impaired production of chemotactic factors by *Pasteurella haemolytica*-infected alveolar macrophages. American Journal of Veterinary Research 43: 285–288.

NICOLET, J. 1990. Overview of the virulence attributes of the HAP-group of bacteria. Canadian Journal of Veterinary Research 54: S12–S15.

ONDERKA, D. K., AND W. D. WISHART. 1988. Experimental contact transmission of *Pasteurella haemolytica* from clinically normal domestic sheep causing pneumonia in Rocky Mountain bighorn sheep. Journal of Wildlife Diseases 24: 663–667.

SHEWEN, P. A., AND B. N. WILKIE. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infection and Immunity 35: 91–94.

SILFLOW, R. M., AND W. J. FOREYT. 1988. Bronchoalveolar lavage technique in bighorn sheep: Use in immediate lungworm diagnosis and cell function tests. Biennial Symposium of the Northern Wild Sheep and Goat Council 6: 98–105.

———, ———, S. M. TAYLOR, W. W. LAEGREID, H. D. LIGGITT, AND R. W. LEID. 1989. Comparison of pulmonary defense mechanisms in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and domestic sheep. Journal of Wildlife Diseases 25: 514–520.

———, ———, AND R. W. LEID. 1993. *Pasteurella haemolytica*, cytotoxin-dependent killing of neutrophils from bighorn and domestic sheep. Journal of Wildlife Diseases 29: 30–35.

SUTHERLAND, A. D., E. GRAY, AND P. W. WELLS. 1983. Cytotoxic effect of *Pasteurella haemolytica* on ovine bronchoalveolar macrophages in vitro. Veterinary Microbiology 8: 3–15.

THORNE, E. T., N. KINGSTON, W. R. JOLLEY, AND R. C. BERGSTROM (editors). 1982. Diseases of wildlife in Wyoming, 2nd ed. Wyoming Fish and Game Department, Cheyenne, Wyoming, 353 pp.

WILD, M. A., AND M. W. MILLER. 1991. Detecting nonhemolytic *Pasteurella haemolytica* infections in healthy Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*): Influences of sample site and handling. Journal of Wildlife Diseases 27: 53–60.

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