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## COMPARISON OF PULMONARY DEFENSE MECHANISMS IN ROCKY MOUNTAIN BIGHORN (*OVIS CANADENSIS CANADENSIS*) AND DOMESTIC SHEEP

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**ABSTRACT:** Alveolar macrophages were obtained from Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and domestic sheep for the purpose of comparing pulmonary host defense mechanisms in the two species. Specific variables studied included (1) characterization of the cell types present in the lung, (2) alveolar macrophage phagocytic and bactericidal functions, (3) measurement of protein levels in lavage fluid, and (4) measurement of cortisol levels in lavage fluid. While phagocytic cell populations were similar between bighorn and domestic sheep, a significantly higher percentage of lymphocytes were present in bighorns than domestics (20% in bighorn versus 6% in domestic sheep). Significant differences were not observed in the phagocytic or bactericidal functions of macrophages between the two species. Significant differences were not observed in either lavage fluid protein levels or in cortisol levels.

**Key words:** Bighorn sheep, *Ovis canadensis canadensis*, domestic sheep, alveolar macrophages, lavage, bactericidal, phagocytosis, cortisol, pneumonia complex, experimental study.

### INTRODUCTION

The pneumonia complex is a major factor affecting bighorn sheep (*Ovis canadensis canadensis*) survival in North America (Potts, 1937; Marsh, 1938; Buechner, 1960; Forrester, 1971; Spraker et al., 1984), and several bighorn sheep populations in North America have been decimated by fatal epizootics of bacterial pneumonia within the past 20 yr (Parks et al., 1972; Thorne et al., 1982; Onderka and Wishart, 1984; Spraker et al., 1984). The pneumonia complex in both bighorn and domestic sheep is a multi-factorial problem including an interaction among bacterial, viral, parasitic and stress factors (Parks et al., 1972; Samson et al., 1987). Although domestic sheep are susceptible to pneumonia, their susceptibility is less than that exhibited by bighorn sheep (Rushton et al., 1979). Indeed, domestic sheep often serve as asymptomatic carriers of the primary bacterial agent, *Pasteurella* spp. (Davies, 1981; Frank, 1982). Circumstantial evidence and controlled experimental studies have indicated that direct contact between the two species may result in fatal pneumonia in bighorn sheep (Fo-

reyt and Jessup, 1982; Coggins, 1988; Onderka and Wishart, 1988; Onderka et al., 1988; Foreyt, 1989).

Stress, either chronic or acute, is a common denominator in the majority of pneumonia-complex deaths. Cortisol, a lipid which is released under conditions of stress, can exert anti-inflammatory activity by suppressing alveolar macrophage (AM) arachidonic acid metabolite release from membrane phospholipids (Flower et al., 1984; Hirata, 1984). Moreover, these effects have been linked to increased disease susceptibility (Hamdy et al., 1964; Roth, 1985). Although serum cortisol levels in bighorn sheep have been measured previously (Spraker et al., 1984; Turner, 1984), we quantitated cortisol levels in bronchoalveolar lavage fluid to determine if differences existed between the two species of sheep in the pulmonary microenvironment.

AM are the host cells primarily responsible for maintaining the sterility of the lower respiratory tract (Trigo et al., 1984; Liggitt et al., 1986). These cells are actively phagocytic, bactericidal and viricidal, and collaborate with lymphocytes in

the production of antibodies against foreign antigens. AM also function in inflammation by releasing membrane-bound arachidonic acid and metabolizing this lipid in response to many inflammatory stimuli. The biological functions of the arachidonic acid metabolites are not completely understood, but it is known that one of these metabolites, leukotriene B<sub>4</sub> has pronounced chemotactic activity for neutrophils and eosinophils, thus providing one of the major stimuli for the migration of polymorphonuclear leukocytes into inflammatory lesions (Leid and Potter, 1985). Furthermore, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) can suppress immune functions and the host inflammatory response by suppressing AM bactericidal capacity (Chensue and Kunkel, 1983; Laegreid et al., 1989) and other AM functions critical in lung defense. Furthermore, 5- and 15-hydroxyeicosatetraenoic acids (HETE's) can function as mucous secretagogues for epithelial cells (Shelhamer et al., 1982; Marom et al., 1983; Harkema et al., 1988). The present study was initiated to identify specific pulmonary factors or circumstances which could result in impairment of host lung defense, a diminution of which would explain why bighorn sheep are more susceptible to pulmonary bacterial infections than are domestic sheep.

## MATERIALS AND METHODS

### Animals

The bighorn sheep used in this study came from two sources. Eleven were from the captive herd maintained on the Washington State University campus (Pullman, Washington 99164, USA). Of these animals, eight were kept in a 2.5 ha pasture, while the remaining three animals were kept in a 7 m × 7 m indoor room. Another 10 animals were from a free-ranging herd located in northeastern Washington and which utilize winter range near Sullivan Lake in Pend Orielle County (48°50'N, 117°15'W). Both rams and ewes were sampled, with the ages for each ranging from 7 mo to 8 yr. Table 1 shows the age and sex classification of the bighorn sheep used to provide samples for each individual variable of this study. Fecal samples were monitored regularly for parasite abun-

TABLE 1. Age and sex classification of bighorn sheep used in analysis of variables.

	7-12 mo	4+ yr
Volume recovered,		
total cells,		
viability	2 males*	7 females
Differential counts	3 males*	7 males, 7 females
Protein	2 males*	7 males, 7 females
Cortisol	2 males*	5 males, 4 females
Killing	2 males*	7 females
Phagocytosis	1 male	6 females

\* Repeated samples collected from these individuals.

dances and animals were considered to have insignificant numbers of *Protostrongylus* spp. larvae throughout the study period. The 19 conventionally-reared domestic sheep used were clinically normal mature Suffolk ewes which had no clinical signs or history of respiratory disease.

The capture of bighorn sheep from the free-ranging herd was accomplished by feeding hay pellets to them within a circular 7 m diameter trap until they became accustomed to the structure. This trapping permitted a minimal induction of stress at the time of capture and a similar structure was used to trap animals from the captive herd. The bighorns kept in the indoor stall could be approached easily and sedation was accomplished using xylazine hydrochloride (Rompun, Miles Laboratories, Bayvet Division, Shawnee, Kansas 66201, USA) in a pole-syringe.

Ten bighorn sheep were used to provide 19 lung lavage samples to be quantitated for cortisol. Six of the samples were collected from six bighorn sheep kept in a 2.5 ha pasture, while one sample was collected from a bighorn sheep from the free-ranging herd. The remaining 12 samples came from three bighorn sheep kept in the indoor 7 m × 7 m room. Two of these animals were lavaged once per wk for 5 wk to provide information on changes in cortisol levels over time.

### Alveolar macrophage collection and processing

Bighorn and domestic sheep were sedated using 0.5 mg/kg of xylazine hydrochloride intramuscularly. Alveolar macrophages (AM) were obtained for study using the lavage method of Silflow and Foreyt (1988), which was a modification of the technique developed by Trigo et al. (1984). A veterinary laryngoscope was used to aid in 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 immedi-

ately 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 administration of 10 mg of yohimbine hydrochloride (Antagonil, Wildlife Laboratories Inc., Fort Collins, Colorado 80525, USA) intravenously. The recovered lavage fluid was centrifuged at 350 *g*, and the cell pellet resuspended in tissue culture medium RPMI-1640 (GIBCO Laboratories, Life Technology Inc., Grand Island, New York 14072, USA) for use in various assays. Cell-free lavage fluid was frozen at  $-70^{\circ}\text{C}$  until protein and cortisol levels were measured. The alveolar cell population was characterized by staining a cytocentrifuge-prepared slide with Dif-Quik Kit (American Scientific Products, McGaw Park, Illinois 60085, USA). Total cell number and viability of each sample were determined by mixing 0.1 ml of cells to 0.1 ml of trypan-blue dye and counting on a hemacytometer. A monolayer was obtained by adherence of the AM to plastic 96-well tissue culture plates. Since neutrophils, lymphocytes and eosinophils did not adhere strongly to plastic they were removed by washing, leaving a population of highly enriched AM.

#### Phagocytosis assay

Alveolar macrophages ( $2 \times 10^5$ ) were suspended with opsonized *Staphylococcus epidermidis* at a ratio of 1:10. Opsonization was accomplished by incubating bacteria in RPMI-1640 containing 10% anti-*S. epidermidis* serum for 30 min at  $37^{\circ}\text{C}$ , then washing the bacteria with RPMI-1640 to remove unbound antibody. The AM/bacteria suspension was incubated at  $37^{\circ}\text{C}$  for 1 hr to allow the cells to phagocytize the bacteria. The percentage of AM with internalized bacteria was counted on Dif-Quik stained and cytocentrifuge-prepared slides of the suspension.

#### Bactericidal assay

The bactericidal assay measures the ability of AM to kill bacteria. The method of Peck (1985) was used. This assay is based on the ability of viable bacteria to reduce a tetrazolium dye, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Opsonized *Staphylococcus epidermidis* were added to a 96-well plate containing a monolayer of AM ( $2.5 \times 10^5$  cells/well) at a ratio of 10 bacteria:AM. After 2 hr incubation at  $37^{\circ}\text{C}$ , the cells were lysed using the detergent saponin. This lysis allowed bacteria which were phagocytized but not killed to be released into the supernatant. Nutrient broth was added to enhance growth of surviving bacteria and the broth was cultured at  $37^{\circ}\text{C}$  for 4

hr. The number of live bacteria was quantified by adding the tetrazolium dye MTT and following its reduction by the surviving and reproducing bacteria. A standard curve was established using eight dilutions of bacteria beginning with a maximum of  $2.5 \times 10^6$ /well. The resulting color change was measured by a Titertek plate reader (Flow Laboratories Inc., McLean, Virginia 22102, USA), and data were communicated to an IBM-PC computer system for analysis. Results were expressed as percentage of bacteria killed during their exposure to AM.

#### Protein levels in lavage fluid

Protein levels were quantitated using a microtiter assay method and BCA commercial reagents (Pierce Chemical Co., Rockford, Illinois 61105, USA). A standard curve was obtained using bovine serum albumin (BSA) as a standard. Fifty  $\mu\text{l}$  of lavage fluid or the BSA standard was placed into wells of a 96-well plate. Four duplicates were used per sample, and 50  $\mu\text{l}$  of 0.15 M phosphate buffered saline (PBS) were added to each well. One hundred fifty  $\mu\text{l}$  of prepared BCA reagent was added to each well and the plate incubated at room temperature for 2 hr. The color change was quantitated using a Titertek plate reader set at a wavelength of 550 nm and the results expressed as mg protein/ml lavage fluid.

#### Cortisol levels in lavage fluid

Lavage fluid samples were purified and concentrated 500-fold by performing a lipid extraction. Twenty-five ml of lavage fluid were mixed with 25 ml of chloroform, vortexed, and centrifuged at 400 *g*. The chloroform fraction was dried under a stream of nitrogen gas and resuspended in 50  $\mu\text{l}$  of ethanol. Ten  $\mu\text{l}$  of concentrated lavage fluid were added to an assay system using a  $^3\text{H}$ -Cortisol RIA kit (Radio Assay Systems Lab Inc., Carson, California 90746, USA) and results were expressed as ng cortisol/ml lavage fluid.

#### Statistics

The Student's *t*-test was used to evaluate differences between experimental groups for the following variables: volume recovered, total cells, viability, protein, cortisol, phagocytosis and killing. The statistical test for comparing two binomial proportions was used to evaluate the differential leukocyte proportions. The relationship between cortisol levels and time between capture and sampling was evaluated using a simple correlation coefficient test. The level of significance was set at  $\alpha = 0.05$ .

TABLE 2. Volume recovered, total cell counts, viability, differential leukocyte proportions, protein and cortisol levels obtained by lung lavage of bighorn sheep and domestic sheep. Data on the percentage of alveolar macrophages with phagocytosed bacteria and percentage of bacteria killed are also reported.

	Bighorn sheep	Domestic sheep
Volume recovered (%)	58.87 ± 2.70 (n = 35)	56.61 ± 3.52 (n = 19)
Total cells (×10 <sup>6</sup> )	12.73 ± 1.26	11.16 ± 1.52
Viability (%)	72.53 ± 4.15	75.20 ± 4.83
Macrophages (%)	73.70 ± 2.95 (n = 30)	81.50 ± 6.07 (n = 8)
Neutrophils (%)	4.70 ± 1.63	11.0 ± 4.14
Eosinophils (%)	2.30 ± 0.77	1.25 ± 0.98
Lymphocytes (%)	19.53 ± 2.83*	6.25 ± 2.44
Protein (mg/ml)	0.505 ± 0.04 (n = 31)	0.569 ± 0.06 (n = 10)
Cortisol (ng/ml)	0.590 ± 0.023 (n = 19)	0.551 ± 0.015 (n = 7)
Phagocytosis (%)	79.29 ± 2.46 (n = 7)	86.00 ± 2.04 (n = 4)
Killing (%)	46.20 ± 8.10 (n = 11)	45.09 ± 5.57 (n = 7)

\* Significantly different ( $P < 0.05$ ).

### RESULTS

A comparison of the cell types recovered from the lungs of bighorn and domestic sheep showed significant differences ( $P < 0.05$ ) between species in the percentage of lymphocytes present (20% in bighorn versus 6% in domestic sheep) (Table 2). The proportions of other cell types present in the lung, including alveolar macrophages (74% in bighorn and 82% in domestic sheep), neutrophils (5%, 11%), and eosinophils (2%, 1%), were not statistically different ( $P > 0.05$ ) between the two groups of animals.

There were no differences between species in the volume of fluid recovered (59 and 57% in bighorn and domestic sheep, respectively), or total cell number ( $13 \times 10^6$ ,  $11 \times 10^6$ ), or viability (73%, 75%) (Table 2).

The ability of AM to function in vitro and phagocytize particles (79 and 86% in bighorn and domestic sheep, respectively) and kill *S. epidermidis* bacteria (46 and 45%) was similar in both species (Table 2). Cells from both bighorn and domestic sheep were actively phagocytic and bactericidal.

The protein (0.505 mg/ml and 0.569 mg/ml in bighorn and domestic sheep) and cortisol (0.590 ng/ml, 0.551 ng/ml) levels

in the lavage fluid were not different between the two species (Table 2). When pulmonary cortisol levels were compared to the time period between capture and sampling, no linear relationship existed as determined by computation of the correlation coefficient ( $r = 0.52$ ). This lack of correlation would indicate that any stress induced by the capture and sampling procedure was insufficient to change the cortisol levels. Cortisol levels measured in two bighorn sheep over a 5 wk period showed individual daily variation but no significant difference ( $P > 0.05$ ) in mean levels between animals (Table 3). No differences in any variable existed between age and sex classifications of the bighorn sheep analyzed in this study.

### DISCUSSION

Bronchoalveolar lavage of bighorn and domestic sheep allowed the recovery of similar numbers and types of cells from the lower respiratory tract of both species. The different cell populations obtained from domestic sheep were similar in number and composition to those reported by Woodside et al. (1983). The number of cells obtained and their viability were less than what is routinely obtained for cattle (Trigo et al., 1984). The reason for this

TABLE 3. Lavage fluid cortisol levels in two bighorn sheep over a 5 wk period.

Week	Cortisol (ng/ml)	
	Bighorn sheep A	Bighorn sheep B
1	0.635	0.684
2	0.607	0.604
3	0.554	0.599
4	0.290	0.644
5	0.561	0.594
Mean $\pm$ SEM	0.529 $\pm$ 0.062	0.625 $\pm$ 0.017

discrepancy is unknown at present. The increased number of lymphocytes observed in bighorn sheep may be a result of several mechanisms. This rise might result from either a subclinical viral infection or from non-infectious particulates. It would be extremely beneficial to know which subpopulations these lymphocytes represented. Increases in the suppressor/helper lymphocyte ratio may well lead to regulation of lung immune responses. Similarly, increases in the helper/suppressor cell ratio may indicate immune complex-mediated lung tissue injury. Both of these phenomena could increase susceptibility to the respiratory pathogens by compromising the pulmonary defense functions of alveolar macrophages or other pulmonary cells.

Alveolar macrophages from bighorn and domestic sheep were able to function in the role of a first-line defense against bacterial invasion as indicated by similar efficiency in phagocytic and bactericidal assays. Protein levels in lavage fluid were quantitated for two reasons: (1) as an indication of vascular permeability in alveolar spaces, and (2) as a means of standardizing the dilution effects of the lavage procedure so cortisol levels could be compared. The observation of similar protein levels in both species of sheep indicated that no differences in vascular permeability occurred at the time of lavage. Furthermore, and contrary to what we expected, there was no correlation between

protein and cortisol levels in lavage fluid. This lack of correlation permitted the comparison of cortisol levels by expressing results as ng/ml of lavage fluid. Two bighorn sheep were monitored over a 5 wk period for changes in cortisol levels. Both bighorns varied in week to week cortisol levels, but there was no significant difference in the mean cortisol levels between the two sheep. Cortisol levels in the lungs of healthy, unstressed bighorn sheep were at low levels, levels which were considered insufficient to predispose these animals to pneumonia. However, under conditions of acute or chronic stress, elevations in cortisol may still be considered as a potential mechanism for the development of respiratory disease.

Protection of bighorn sheep populations from the devastating effects of the pneumonia complex depends on understanding the mechanisms involved in the disease process. This study indicates that bighorn sheep have intact respiratory host defense functions including AM bactericidal and phagocytic ability. Furthermore, these functions are similar to those of domestic sheep.

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