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BRONCHOALVEOLAR LAVAGE TECHNIQUE IN BIGHORN SHEEP: USE IN IMMEDIATE LUNGWORM DIAGNOSIS AND CELL FUNCTION TESTS

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Abstract: Segmental lavage was performed on 6 sedated Rocky Mountain bighorn sheep (Ovis canadensis canadensis) using fiberoptic bronchoscopy. Alveolar cells were recovered for characterization and function tests. Adult lungworms (Protostrongylus spp.) were observed during the lavage procedure in the lavage fluid recovered from 3 of 6 sheep. Microscopic observation of the lavage fluid revealed the presence of Protostrongylus spp. larvae and eggs in 5 of the 6 sheep. Alveolar cell viability was severely reduced in cases where larvae were numerous, and a direct correlation between numbers of larvae and cell death was observed ($r = 0.80$). The bronchoalveolar lavage technique is effective for immediate diagnosis of lungworms in bighorn sheep and may be more accurate than existing methods. In addition, bronchoalveolar lavage is a tool for collecting alveolar cells for the purpose of studying the defense mechanisms of the lung at the cellular level.

The pneumonia complex is a major mortality factor affecting bighorn sheep survival in North America (Potts 1937, Marsh 1938, Buechner 1960, Forrester 1971, Spraker et al. 1984). Its pathogensis is multifactorial and complex.

Several factors have been implicated in the role of predisposing bighorn sheep to opportunistic bacteria, of which Pasteurella hemolytica is the most deadly (Parks et al. 1972, Thorne et al. 1982, Onderka and Wishart 1984). The role of host defense at the cellular level is an area where little research has been focused to date. It involves investigation of alveolar cell types to understand their function in healthy animals, and to observe mechanisms involved in the impairment of their function during disease conditions.

The first line of defense for the lung is provided by the alveolar macrophage (Trigo et al. 1984, Liggitt et al. 1986). Macrophages function to phagocytize and kill bacteria and viral particles, and interact with lymphocytes to play a vital role in subsequent antibody production against foreign antigens. The alveolar macrophage can routinely be collected from the lungs using bronchoalveolar lavage technique. They can be maintained in tissue culture for at least 1 week, which enables an investigator to reconstruct parasitic, viral, or bacterial infections in tissue culture conditions, and to evaluate the effects on host defense parameters.

An area of active research in humans and domestic animals is the role of inflammatory chemical mediators in disease. Phagocytic cells release membrane-bound arachidonic acid and synthesize its metabolites in response to inflammatory stimuli. Different inflammatory cell types from various tissue sites and species generate characteristic profiles of arachidonic acid metabolites following cellular stimulation. These metabolites have critical lung defense roles when held in balance, yet can also result in tissue injury when that balance is lost (Taylor, 1986). This tissue injury can occur in the lung and predispose alveolar lining cells to viral and bacterial infection. The functions of the inflammatory mediators include chemotactic activity on neutrophils and eosinophils, thus providing one of the major stimuli for the migration of polymorphonuclear leukocytes into inflammatory lesions. They also play a role in causing several immune functions to become depressed and thus predispose a host to secondary infection. This is believed to be one of the mechanisms by which respiratory viruses such as parainfluenze-3 acts in the pathogenesis of the pneumonia complex (Laegreid et al. unpublished). The information gained from studying these mediators may suggest reasons for differences in species susceptibility to the pneumonia complex and may provide methods for prevention of compromised lung defenses in bighorn sheep. Understanding the role of inflammatory mediators has led to the development of such pharmacological agents such as corticosteriods or steriod-like products having anti-inflammatory action. An example of beneficial response from such agents is seen in the use of cyclooxygenase inhibitors to reverse the virus induced dysfunction of macrophages (Sestini et al. 1984, Rinaldo et al. 1985, Laegreid et al. 1988).

Parasitic infection is 1 of the factors which leads to impairment of host lung defense. This study confirms the role of Protostrongylus spp. in impairment of macrophage function by a toxic effect observed on macrophages obtained via bronchoalveolar lavage.

Contact between domestic sheep and bighorn sheep has proven to be detrimental under controlled experimental conditions (Foreyt 1988) and in some cases may have resulted in major die-offs, due to pneumonia, in wild bighorn herds (Foreyt and Jessup 1982, Coggins 1988). It is assumed that such contact resulted in a transfer of Pasteurella hemolytica from domestic sheep to bighorn sheep. The question to be answered is why bighorn sheep are so much more susceptible to the serotypes of Pasteurella hemolytica than are domestic sheep.

Identification of factors or circumstances leading to impairment of host lung defense would perhaps explain why bighorn sheep are more susceptible to pulmonary bacterial infections than domestic sheep. This would permit development of specific remedies which may be immunological, pharmacological or managerial.

METHODS

Pulmonary Phagocyte Collection And Processing

Alveolar macrophages were isolated from the lungs of 6 bighorn rams, 3-6 years old, following sedation with 50 mg of xylazine hydrochloride (Rompun, Miles Laboratories, Bayvet Division, Shawnee, KA 66201, USA)

intramuscularly. The herd of bighorns used in the study was located on their winter range on Hall Mountain in northeast Washington. The trapping procedure involved minimal stress to the rams which were accustomed to being fed inside the trap enclosure. A fiberoptic bronchoscope (Machida, Orangeburg, NY, 10962, USA) with an insertion tube 145 cm long and diameter of 6 mm was gently lodged into the caudal lobe of the lung. Aliquots of sterile physiological saline, 60 cc, were infused and immediately withdrawn. A total of 300 cc of fluid was instilled of which 40-50% was recovered. This lavage fluid was placed in 250 cc centrifuge tubes and kept on ice for three hours prior to analysis at the lab. The alveolar cell population was characterized by staining a cytocentrifuge prepared slide with Dif-Quik stain. Total cell number and viability of each sample was obtained using a trypan-blue exclusion method in which 0.1 ml of cells were added to 0.1 ml of trypan-blue dye and counted on a hemocytometer. Dead cells stain blue due to their inability to exclude the dye. The cells were pelleted by centrifugation at 150 x g, and resuspended in nutritional media (RPMI) for use in various assays. A monolayer of adherent cells was obtained by placing them on plastic 6-well tissue culture plates.

Since cell viability was low from the 6 rams, we decided to use 4 captive bighorn ewes ages 2-6 located at Washington State University for a source of alveolar cells to use in phagocytosis and bactericidal assays. These animals were trapped and sedated in the same manner described above for the 6 rams, and the phagocyte collection and processing procedure was identical. No Protostrongylus adults or larvae were recovered during lavage of these 4 ewes.

We routinely administer antibiotics (5 ml LA-200) at the time of bronchoscopy as a preventative measure against potential infection caused by handling the animals. We reversed the effects of the xylazine by giving 10 mg of yohimbine hydrochloride (Antagonil, Wildlife Laboratories Inc., P. O. Box 8938, Fort Collins, CO, 80525, USA) intravenously.

Phagocytosis Assay

Alveolar cells (2 x 10^5) were suspended along with Staphyloccocus epidermidis at a ratio of 1:10. This suspension was incubated at 37 C for 1 hour to allow the cells to phagocytize the bacteria. The percent of alveolar cells with internalized bacteria were counted on Dif-Quick stained, cytocentrifuge prepared slides of the suspension.

Bactericidal Assay

The bactericidal assay measures the ability of phagocytes to kill bacteria. The method of Peck (1985) which is based on the ability of viable bacteria to reduce a tetrazolium dye, MTT, was used. Alveolar cells were allowed to adhere to 96-well tissue culture plates. To this monolayer was added a 10:1 ratio Staphyloccocus epidermidis. After 2 hours incubation at 37 C, the cells were lysed using the detergent saponin. This allowed the release into the supernatant of bacteria which may have been phagocytized but not killed by the cells. Nutrient broth was added to enhance growth of surviving bacteria for 4 hours. The number of live bacteria was then quantified by adding the tetrazolium dye MTT

which was reduced by reproducing bacteria. The resulting color change wasmeasured by a Titertek plate reader, and data were communicated to an IBM-PC for analysis. Results were expressed as percent bacteria killed during the exposure to the alveolar cells.

Lungworm Larvae Numbers

Protostrongylus spp. larvae were observed in the tissue culture plates using an inverted microscope. The number of larvae were counted and expressed as total number of larvae per lavage. Once counted, the larvae were separated from the adherent alveolar macrophage population by washing the tissue culture plates with RPMI.

RESULTS AND DISCUSSION

Use of the bronchoalveolar lavage technique was successful in harvesting alveolar cells of the lower respiratory tract. The animals recovered rapidly from sedation after receiving yohibmine hydrochloride, and no adverse effects from the procedure were observed. An average of 44% of the fluid used in the lavage was recovered and contained an average of 7.9×10^6 cells (Table 1). As lavage fluid was being retracted, adult Protostrongylus spp. were observed grossly; eggs and larvae were observed microscopically on tissue culture plates. Direct observation plus the capability to quantify parasites in lavage fluid provided a more accurate assessment of pulmonary airway invasion by Protostrongylus than fecal examination methods.

Table 1. Summary data for alveolar cells and Protostrongylus recovered during lung lavage from 6 rams.

Tag #	Fluid Recovered (%)	Total Cells ($\times 10^6$)	Viability (%)	Protostrongylus	
				Larvae	Adults
Or-12	32.50	5.10	88	0	No
Y-15	51.67	7.75	84	10	Yes
Y-12	43.33	7.75	13	30	Yes
G-400	46.67	8.75	29	50	Yes
Y-11	40.00	12.50	10	30	No
Or-277	45.00	5.50	59	15	No
Mean $\pm$ SE	44.03$\pm$2.0	7.89$\pm$1.09	47.17$\pm$14	22.5$\pm$7.27	

The lavage technique can also be accomplished without a fiberoptic bronchoscope. A 145 cm plastic tube 6 mm diameter has been successfully placed in the lung of sedated bighorn sheep with the aid of a veterinary laryngoscope. The advantages of this method include greatly reduced cost of equipment, and elimination of the need for electricity. The fiberoptic

scope however, offers the advantage of allowing visualization of the airways for the presence of parasites or other disease conditions.

A wide range of cell viability was observed using trypan-blue exclusion (10-88%). The percent of dead cells correlated with heavy loads of Protostrongylus larvae (r = 0.80) (Fig. 1). This suggests a toxic effect of the parasite on the alveolar cells which may be responsible for predisposing an animal to opportunistic bacteria commonly seen in the pneumonia complex.

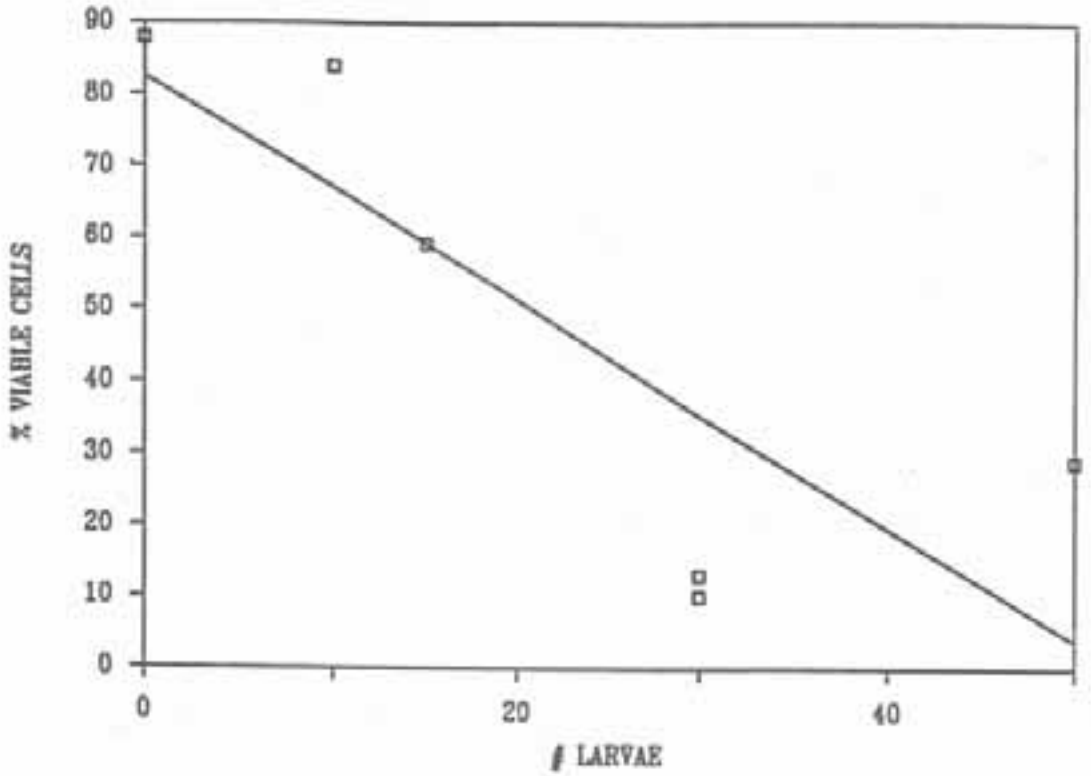

Figure 1. Correlation line (r = 0.80) showing effect of Protostrongylus larvae on alveolar macrophage viability (n = 6 rams).

The cell types present in the bighorn sheep lung were characterized and consisted of an average of 77% macrophages, 14% neutrophils, and 9% lymphocytes (Table 2).

Due to poor viability of alveolar cells from the 6 bighorn rams, the cells from 4 captive bighorns were analyzed for ability to phagocytize and kill bacteria. When bacteria were present at 10:1 bacteria per alveolar cell, 83% of the cells were considered actively phagocytic and 58% of the bacteria were killed in 2 hrs (Table 3).

Results from Table 3 indicate that the live cells are capable of functioning in the role of first-line defense against bacteria. However, when the total number of live cells is decreased by toxic agents such as

Table 2. Differential cell counts from alveolar cells recovered by lung lavage from 6 rams.

Tag #	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)
Or-12	86	5	9
Y-15	82	11	7
Y-12	65	15	20
G-400	50	47	3
Y-11	98	2	0
Or-277	80	2	18
Mean ± SE	76.83±6.9	13.67±6.99	9.5±3.27

Table 3. Phagocytosis and bactericidal ability of alveolar cells from lung lavage of 4 captive ewes. No Protostrongylus were present in the lungs of these animals.

Tag #	Phagocytosis (%)	Bactericidal (%)
28	79.50	76.92
291	77.00	74.67
39	91.50	46.78
369	82.00	32.20
Mean ± SE	82.5 ± 3.17	57.64 ± 10.9

Protostrongylus, the ability of the host to defend against secondary organisms is seriously compromised.

By applying the technique of bronchoalveolar lavage in bighorn sheep, two major purposes can be accomplished: 1) alveolar cells can be obtained for in vitro studies relating to host defense mechanisms, and 2) immediate diagnosis of lungworms is possible by the observation of adults in the airways or retrieved fluid, or by examination of the fluid for eggs and/or larvae. Furthermore, the correlation of the effects of parasite load in the lung with cell function can be made. In addition, many more of the factors involved in the pathogenesis of the pneumonia complex can be studied in an in vitro environment, thus eliminating costly and destructive waste of valuable live animals necessary when in vivio experiments are performed.

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