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Pasteurella haemolytica Lipopolysaccharide-Associated Protein Induces Pulmonary Inflammation after Bronchoscopic Deposition in Calves and Sheep

K. A. BROGDEN,[1]* M. R. ACKERMANN,[1] AND B. M. DEBEY[2]

Respiratory Disease Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010,[1] and Veterinary Diagnostic Laboratory, University of California, Tulare, California 93274[2]

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The lipopolysaccharide (LPS)-associated protein (LAP) was extracted from *Pasteurella haemolytica* serotype A1 strains L101 (bovine origin) and 82-25 (ovine origin). Extracts contained 0.017% total LPS and appeared as only two bands at 14 and 16.6 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To determine the extent of pulmonary inflammation induced by LAP and its possible role in the pathogenesis of pneumonic pasteurellosis, LAP (500 μg in pyrogen-free saline [PFS]) was deposited by fiber-optic bronchoscopy into the dorsum of the caudal portion of the cranial lobe of the right lung of calves (strain L101 LAP) and sheep (strain 82-25 LAP). LPS (500 μg in PFS), 3-h *P. haemolytica* cultures (1.6×10^8 to 1.9×10^8 CFU in PFS), and PFS alone were deposited similarly as controls. At necropsy, 24 h after deposition, gross and histologic pulmonary lesions of calves and sheep given LAP, LPS, and *P. haemolytica* were similar and consisted of various degrees of acute bronchopneumonia (relative severities of lesions induced: LAP < LPS < live organisms). By subjective histologic interpretation and semiquantitative morphometry, animals given LAP had the highest percentage of macrophages per alveolar lumen and the lowest percentage of neutrophils. The lesions from animals given LPS were more severe than those given LAP, but the morphometric cell counts were similar. In contrast, animals inoculated with *P. haemolytica* had lesions typical of this agent, consisting of many neutrophils, proteinaceous exudate, and a few macrophages. Morphometrically, these lesions had the highest numbers of neutrophils and the lowest numbers of macrophages. These studies show that LAP can induce an inflammatory response in the alveolar lumens and may play a role in the pathogenesis of pneumonic pasteurellosis.

The outer membrane of the gram-negative bacterial cell envelope contains primarily lipopolysaccharide (LPS), porin and other proteins, and phospholipids. During periods of in vitro or in vivo growth, large sections of the outer leaflet of the outer membrane are released into the extracellular environment as membranous vesicles or endotoxins. The proteinaceous component of endotoxin, called endotoxin protein or LPS-associated protein (LAP), is heterogeneous, contains up to 12 proteins, and varies in size from 10 to 35 kDa (10, 11, 13) depending upon the culture conditions, the strain of organism, or the method of extraction. Often, more than one protein is present when LAP is extracted with solvents (e.g., *n*-butanol and trichloroacetic acid, etc.) from whole bacterial preparations. Only one LAP coextracts with rough LPS by the phenol-chloroform-petroleum method of Galanos et al. (9). LAP is then recovered from the phenol (void of LPS) by the isobutanol and water method of Strittmatter and Galanos (19). The LAP, extracted from *Escherichia coli*, *Salmonella minnesota*, and *Yersinia enterocolitica*, has an apparent molecular mass of 36 to 37 kDa and an amino acid composition similar to that of the OmpC and OmpF porin proteins of *E. coli* (17, 19).

The role of LAP, as a component of endotoxin, in the pathogenesis of pneumonic pasteurellosis is not known. Any biological activity of LAP in endotoxin would be masked by the potent inflammatory capability of the LPS moiety (14). The purpose of this study was to determine the extent of pulmonary inflammation induced by LAP extracted from *Pasteurella haemolytica* serotype A1 (of bovine and ovine origin) in calves and sheep. The severity and type of lesion were then compared with that induced by LPS or live organisms deposited similarly.

* Corresponding author. Mailing address: USDA-ARS-National Animal Disease Center, 2300 Dayton Rd., P.O. Box 70, Ames, IA 50010. Phone: (515) 239-8593. Fax: (515) 239-8458.

MATERIALS AND METHODS

Animals. Eight 5.0-month-old Jersey bull calves (range, 4.7 to 5.4 months) and seven 30.5-month-old Columbia cross-bred rams (range, 29.2 to 31.0 months) were used. All animals were from conventional herds or flocks reared outdoors in open-front sheds. For this study, the animals were put into isolation rooms and treated by methods approved by the American Association for Accreditation of Laboratory Animal Care.

Bacteria and bacterial extracts. *P. haemolytica* serotype A1 bovine strain L101 and ovine strain 82-25 were used. Organisms for bronchoscopic deposition were grown in tryptose broth for 3 h at 37°C and adjusted to a standard concentration as described previously (12). Inocula, suspended in pyrogen-free saline (PFS; Baxter Healthcare Corp., Deerfield, Ill.), contained 1.6×10^8 CFU/ml (strain L101) and 1.9×10^8 CFU/ml (strain 82-25).

Organisms for extraction of LPS and LAP were grown on blood agar base infusion agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.), washed, and dried (4). Cells from strain L101 (6.0 g [dry weight]) and strain 82-25 (8.7 g [dry weight]) were prepared. Rough LPS was extracted (in 1.0-g lots) with a 50-ml extraction mixture containing phenol, chloroform, and petroleum ether (2:5:8) as described by Galanos et al. (9). The LPS precipitate was washed three times with 80% (wt/vol) phenol and three times with ethyl ether and air dried at room temperature. LPS was suspended in pyrogen-free distilled water (PFW; Baxter Healthcare), heated at 56°C for 1 h, and lyophilized. To remove residual protein, LPS (1.5 mg/ml) was suspended in PK digestion buffer containing 10 mM Tris buffer and 145 mM NaCl (pH 7.2) with 0.2% sodium dodecyl sulfate (SDS) and 27.5 U of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml, heated at 60°C for 2 h, and incubated overnight at 37°C. LPS was precipitated

with saturated sodium acetate and absolute ethanol (20:2:180) at room temperature, and the digestion and precipitation procedures were repeated. The final precipitate was suspended in PFW and pelleted by ultracentrifugation at 100,000 × g for 20 h at 4°C, suspended in PFW, and lyophilized.

LAP was extracted from the discarded phenol supernatant of the rough LPS extraction procedure as described by Strittmatter and Galanos (19) with the following modifications. PFW (0.4 ml) was added to the discarded phenol supernatant to precipitate any residual LPS not removed during the initial LPS precipitation procedure. The phenol solution was centrifuged at 3,020 × g for 15 min at 4°C. The phenol was removed, mixed with water-saturated isobutanol and water (1:1.73:0.2), and stirred for 1 h at room temperature. The mixture was transferred to a glass conical centrifuge tube and partitioned by centrifugation at 300 × g for 15 min at 4°C. The phenol, isobutanol, and water were removed, allowing the interphase protein to stick to the tube wall. The protein was resuspended in 90% (wt/vol) phenol. Water-saturated isobutanol and water were added to the phenol mixture in the ratio described above, mixed, and partitioned by centrifugation. After two such washes, the protein interphase was washed in situ on the tube wall three times with ethanol and three times with ethyl ether and air dried. LAP was resuspended in PFW and lyophilized.

Chemical analysis. Total protein was determined by a protein assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin (Sigma) as the standard. Total LPS was determined with the Quantitative Chromogenic Limulus Amebocyte Lysate Assay (Whittaker Bioproducts, Inc., Walkersville, Md.) with L101 or 82-25 LPS as the standard. LAP extracts were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Parsons et al. (15) with a 6.0% stacking gel over a 12.5% resolving gel.

Serology. Previous exposure of calves and sheep to *P. haemolytica* serotype A1 was determined with an enzyme-linked immunosorbent assay by measuring serum antibody titer to a whole-cell lysate (100 μl of a lysed suspension containing 1.0×10^8 CFU/ml prepared in 0.05 M Tris buffer [pH 7.0] as described previously [5]), LPS (100 μl of a solution containing 10 μg of LPS per ml in 0.1 M carbonate buffer [pH 9.6]), and LAP (100 μl of a solution containing 10 μg of LAP per ml in 0.14 M NaCl with 10 mM sodium phosphate buffer [pH 7.2]) in styrene plates (Immulon 1; Dynatech Laboratories, Inc., Chantilly, Va.) as described previously (18). Bound antibody was detected with peroxidase-labelled rabbit anti-ovine immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) or peroxidase-labelled sheep anti-bovine immunoglobulins G1 and G2 (Bethyl Laboratories, Inc., Montgomery, Tex.) and tetramethylbenzidine (Kirkegaard & Perry Laboratories) or *o*-phenylenediamine dihydrochloride (Sigma).

Fiber-optic bronchoscopy. Bronchoscopy was performed in nonintubated, sedated sheep and calves via the nasotracheal route as described previously (3, 4). The bronchoscope (model VFS-2; Schott Fiber-optics, Inc., Southbridge, Mass.) was inserted into the dorsum of the caudal portion of the cranial lobe of the right lung (pulmonary deposition site) in each animal for deposition of inocula. Two calves were given 5 ml of a sterile 500-μg/ml suspension of L101 LAP in PFS, two calves were given 5 ml of a sterile suspension of L101 LPS in PFS, and two calves were given 5 ml of sterile PFS. The remaining two calves were given 5 ml of a culture containing 1.6×10^8 CFU of L101 per ml of PFS.

Two sheep were given 5 ml of a sterile 500-μg/ml suspension of strain 82-25 LAP in PFS, two sheep were given 5 ml of a sterile suspension of strain 82-25 LPS in PFS, and one sheep was given 5 ml of sterile PFS. The remaining two sheep were given 5 ml of a culture containing 1.9×10^8 CFU of strain 82-25 per ml of PFS.

Necropsy. At 24 h postdeposition, all animals were euthanized with pentobarbital and exsanguinated. At necropsy, the lungs were evaluated grossly, and pieces of tissue were taken from the pulmonary deposition site and fixed in 10% neutral buffered formalin solution, dehydrated, cleared, embedded in paraffin, sectioned, and stained with Giemsa and hematoxylin and eosin stains. Pieces of tissue were cultured on 0.5% defibrinated sheep blood in Trypticase soy agar, on MacConkey agar, and in Hayflick's medium containing 10% yeast autolysate and 20% horse serum (both with and without 0.7% agarose) as described previously (6). Isolated bacterial species were identified by conventional methodology (7).

Tissue samples for immunocytochemistry to CD68 and cytokeratin for morphometric analysis were immersion fixed in neutral-buffered 10% zinc formalin, dehydrated, cleared, embedded in paraffin, and sectioned onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, Pa.). Sections were stained with a double labelling technique incorporating a stain to label macrophages (anti-CD68) and a stain to label alveolar lining cells (anti-cytokeratin). The cytokeratin stain outlines the borders of alveolar lumens (and also the epithelium of bronchi and bronchioles), allowing definition of the border of alveolar septae and lumens. This technique clearly defines the boundaries of the alveolar wall and allows accurate counts of intraseptal and intraluminal neutrophils.

The CD68 and cytokeratin double labelling procedure follows a protocol for CD68 immunoreactivity developed in our laboratory with a few modifications (1). Briefly, staining for CD68 involves mouse anti-CD68 primary antibody (EBM11; Dako Corp., Carpinteria, Calif.) at a 1:25 dilution (37°C) and secondary biotinylated goat anti-mouse antibody for 30 min, followed by an avidin-peroxidase solution. The chromogen for CD68 is a metal-enhanced 3′,3-diaminobenzidine solution (Immunopure; Pierce Chemical Co., Rockford, Ill.) that creates a brown precipitate. Slides were then treated for cytokeratin immunoreactivity with anti-cytokeratin (clone MNF116; Dako; specific for cytokeratins 5, 6, 8, 17, and 19) at a 1:25 dilution followed by biotinylated goat anti-mouse

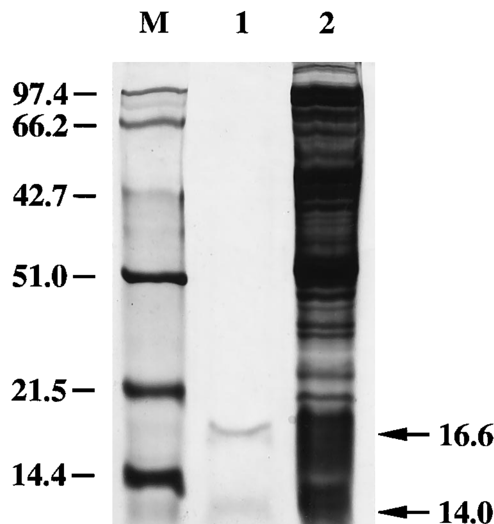

FIG. 1. SDS-PAGE pattern of *P. haemolytica* 82-25 LAP in 12.5% polyacrylamide gel in 25 mM Tris–192 mM glycine–0.01% SDS buffer (pH 8.3) at 100 V/gel for 3 h. Lanes: M, 14.4- to 97.4-kDa molecular mass markers; 1, LAP; 2, a whole-cell lysate. Extracts of LAP were relatively pure and consisted of two bands at 14 and 16.6 kDa (arrows indicate bands in center lane).

antibody, streptavidin-phosphatase, and phosphatase chromogen (Histomark Red; Kirkegaard & Perry Laboratories), which results in a red precipitate. Sections were counterstained with Harris hematoxylin. Control sections lacked primary antibody (EBM11/MNF116), or the primary antibodies were substituted with irrelevant antibodies.

The limited morphometric studies done were semiquantitative in an attempt to measure the relative influx of neutrophils and macrophages within alveolar lumens and septae. This technique is based on a protocol developed in a previous study. Two sections of lung from each inoculated site were examined, and at least four alveoli per lung lobe in the areas of the most intense inflammation were chosen for morphometric measurements. This resulted in counts of eight alveoli per inoculum. Measurements for alveoli included the following: (i) the number of neutrophils or macrophages in alveolar lumens, (ii) the area of the alveolar septa, (iii) the area of the alveolar lumen, and (iv) the total area of the alveolar unit.

Counts were made on an image analysis system containing a Zeiss Axioplan microscope, a 40× NEOFLUR objective, and an Optronics CCD video camera system. Images were transmitted to a Sony PVM 1343 MD color monitor. Image processing of selected fields, including enhancement, manual tracing, point counts, and area of calculation, were performed with Vidas 2.1 software (Kontron Elektroniks). Images are 512 by 480 pixels.

Statistics. Differences among the pulmonary inflammatory cell response of animals given LAP, LPS, or live organisms were determined with the Statistical Analysis System (SAS Institute Inc., Cary, N.C.).

RESULTS

LAP analysis. The LAP extract from strain 82-25 contained 0.017% LPS and appeared as two bands at 14 and 16.6 kDa after SDS-PAGE (Fig. 1). The LAP extract from strain L101 appeared as only one band. The overall yield of LAP varied from 2.2 mg (0.03%) for strain 82-25 to 2.3 mg (0.04%) for strain L101.

Serology. Calves had antibody titers to strain L101 lysate ($\log_2$ titer, 8.5 ± 0.3 [mean ± standard error]), LPS ($\log_2$ titer, 9.2 ± 0.2), and LAP ($\log_2$ titer, 7.8 ± 0.3), indicating previous natural exposure. Ruminants have *P. haemolytica* as part of their normal flora. Likewise, sheep had antibody titers to strain 82-25 lysate ($\log_2$ titer, 9.3 ± 0.5), LPS ($\log_2$ titer, 9.6 ± 0.4), and LAP ($\log_2$ titer, 7.9 ± 0.6).

Necropsy. Gross pulmonary lesions for calves and sheep given LAP, LPS, and *P. haemolytica* were similar and consisted

of various degrees of acute bronchopneumonia. Two calves given LAP and one sheep (which died after anesthesia) had mild to moderate lesions at the pulmonary deposition site. The two calves and one sheep given LPS had moderate lesions; the remaining sheep had minimal gross lesions. All cattle and sheep inoculated with *P. haemolytica* had moderate to severe lesions.

Histologically, lungs from the two calves given LAP contained moderate infiltrates of macrophages and neutrophils within alveolar lumens and moderately dilated bronchi and bronchioles (Fig. 2A). Similar lesions were present in calves given LPS (Fig. 2B). In contrast, lung regions inoculated with *P. haemolytica* L101 had lesions typical of this agent, consisting of dense and numerous neutrophils within many dilated alveolar lumens, bronchi, bronchioles, and interlobular septae and large regions of necrosis (Fig. 2C). These infiltrates also contained large amounts of seroproteinaceous fluid and fibrin. By subjective histologic interpretation and semiquantitative morphometry, the calves given LAP had the highest numbers of macrophages per alveolar lumen and the lowest numbers of neutrophils (Table 1). The lesions from calves given LPS were similar to those given LAP. The two calves given *P. haemolytica* L101 had 9.2×10^1 and 1.8×10^7 CFU/g of lung tissue. Mycoplasmas were not isolated. Infected tissue differed markedly in histopathologic features because of the dense infiltrates of neutrophils, fibrin, necrosis, and proteinaceous exudate. Morphometrically, these lesions had the highest numbers of neutrophils with the fewest numbers of macrophages (Table 1). In addition, the two calves inoculated with LAP had marked, multifocal accentuation of the bronchi and bronchioles as a result of dense adventitial infiltrates of lymphocytes and plasma cells. A similar finding was present, to a lesser degree, in one calf inoculated with *P. haemolytica.*

Histologically, lesions in the sheep lungs were similar to those in the cattle. Only one sheep given LAP was included in the analysis (the other died after anesthesia). By morphometric analysis, trends were present similar to those in lesions seen in cattle: lesions in the sheep given LAP and LPS were similar and had relatively high numbers of macrophages and fewer neutrophils (Table 1). The two sheep given *P. haemolytica* 82-25 had 2.6×10^5 and 3.1×10^6 CFU/g of lung tissue. Mycoplasmas were not isolated. Infected tissue also had extensive infiltrates of neutrophils, seroproteinaceous exudate, and fibrin and fewer numbers of macrophages.

DISCUSSION

With the technique for isolating LAP from other gram-negative bacteria, LAP could be isolated with rough LPS from *P. haemolytica*. While there are some similarities in the general properties of the *P. haemolytica* LAP and those of other bacteria, there are also some important differences. For example, like other endotoxin proteins, *P. haemolytica* LAP was isolated in a low yield, 0.04 to 0.05% (19). The LAP was also hydrophobic and difficult to resuspend in aqueous buffers (11). However, unlike other preparations, the *P. haemolytica* LAP contained only one to two bands after SDS-PAGE, and they were considerable smaller in size than those in other preparations (10, 11, 16, 17). Only two reports describe an endotoxin protein of about 12 kDa (13, 24).

The immunomodulatory properties of endotoxin proteins are well known. They can activate the proliferation of B cells (10, 11, 13, 21), stimulate polyclonal antibody synthesis (20), activate tumoricidal macrophages (20), and induce interferon activity in mice (20). Endotoxin protein can also act as both an adjuvant (20) and an immunogen (8). The role of LAP, as a component of endotoxin, in the pathogenesis of disease is not known. Any biological activity of LAP in endotoxin would be masked by the potent inflammatory capability of the LPS moiety (14).

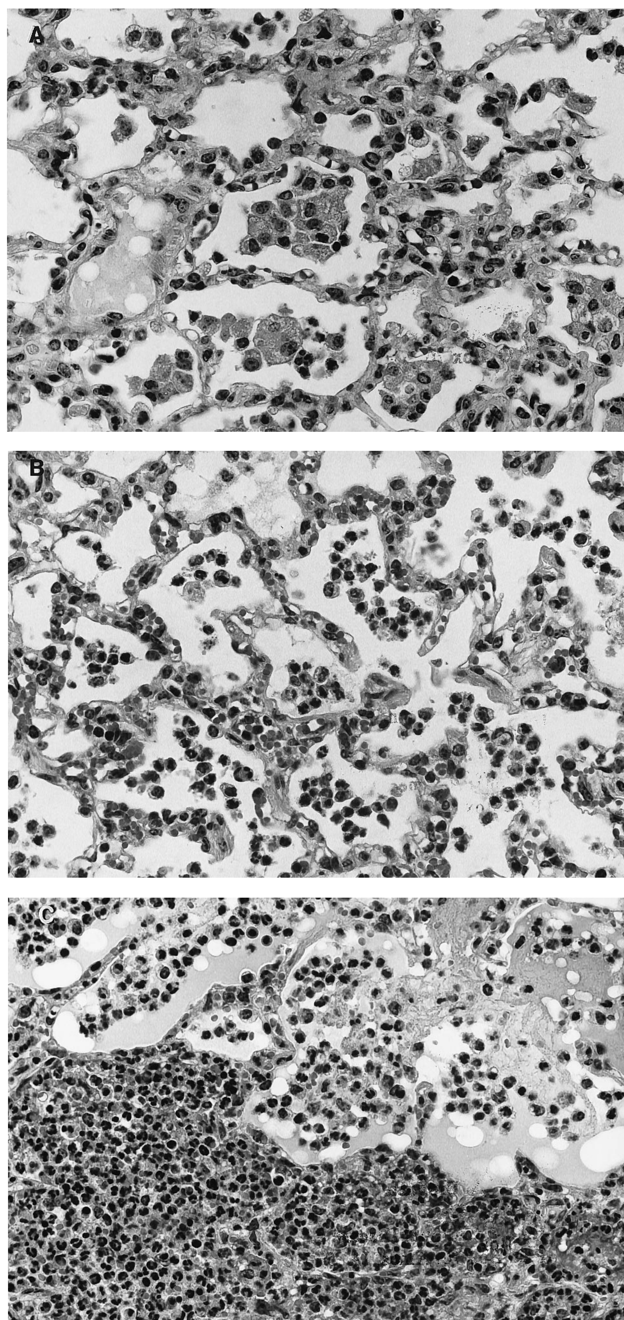

FIG. 2. Micrographs of representative lesions in lungs of calves inoculated with either *P. haemolytica* LAP (A), *P. haemolytica* LPS (B), or live *P. haemolytica* (C) by fiber-optic bronchoscopy. (A) Few neutrophils and numerous macrophages with abundant cytoplasm are visible in alveolar lumens in the lungs of calves inoculated with LAP. (B) Similar lesions are present in the lungs of calves inoculated with LPS (B), but there are fewer macrophages. (C) Alveolar lumens in the lungs of calves inoculated with live *P. haemolytica* have dense infiltrates of numerous neutrophils typical of natural and experimental pneumonic pasteurellosis. Magnification, ×30. Stained with hematoxylin and eosin.

The moderate neutrophil infiltrates in the alveolar lumens

TABLE 1. Semiquantitative morphometric analysis of cells in the lungs from cattle and sheep inoculated with either *P. haemolytica* LAP, *P. haemolytica* LPS, or live *P. haemolytica* (PH) by fiber-optic bronchoscopy[a]

Animals	Treatment	Alveolar area (μm^2)[b]	No. of cells (mean ± SE)	
			Neutrophils	Macrophages
Calves	LAP	5,300 ± 541	21.8 ± 3.1	4.5 ± 1.2
	LPS	5,775 ± 143	18.8 ± 2.5	4.3 ± 1.8
	PH	4,605 ± 134	26.4 ± 4.1	3.2 ± 1.5
Sheep	LAP	4,923[c]	16.6	4.8
	LPS	3,701	23.9	2.1
	PH	4,678 ± 257	39.5 ± 12.3	0.6 ± 0.1

[a] Lung tissues were stained with a double-labelling immunocytochemical technique incorporating a stain to label macrophages (anti-CD68) and a stain to label alveolar lining cells (anti-cytokeratin). Neutrophils were recognized by their typical morphology. Two sections of lung from each inoculated site were examined, and at least four alveoli per lung lobe in the areas of the most intense inflammation were chosen for morphometric measurements. No significant differences were present in these semiquantitative analyses. Calves and sheep inoculated with PFS had fewer than one macrophage per alveoli and no neutrophils. Trends indicated higher numbers of macrophages and lower numbers of neutrophils in the lungs of animals inoculated with LAP and LPS and lower numbers of macrophages and higher numbers of neutrophils in animals inoculated with *P. haemolytica.*

[b] Mean ± standard error.

[c] Only one animal per group.

and moderately dilated bronchi and bronchioles of the two calves and sheep given LAP may not be due to LAP but may be due to the low level of LPS (0.017%) in the LAP preparation. Similar lesions, although more extensive, were present in calves and sheep given LPS. LPS is known to induce neutrophil infiltrates in respiratory airways (4).

The monocytic and lymphocytic peribronchiolar response and the increased numbers of macrophages in the alveolar lumens of calves and sheep given LAP were interesting and unexpected findings. These types of responses are usually seen during mycoplasma infection. However, mycoplasmas were not isolated from any pulmonary tissues taken from the deposition site. Similar results were seen in a preliminary study of sheep given LAP from *P. haemolytica* 82-25 (4a). Perhaps the immunomodulatory properties of the extract can account for the monocytic and lymphocytic response at the deposition site. Whether the increased influx of macrophages into the alveolar lumen would eventually lead to the characteristic zone of spindle-shaped, basophilic macrophages oriented to form whorls and parallel bundles of cells that give the characteristic appearance of streaming in clinical disease (2, 12) is not known at this time.

LAP is one of a variety of *P. haemolytica* components that can induce damage when deposited into the lungs of ruminants that include LPS (4, 23), capsular polysaccharide (3), and leukotoxin (22, 23). LPS is highly inflammatory and induces fibrinopurulent inflammation with edema, hyperemia, hemorrhage, and focal necrosis of alveolar epithelium (4, 23). Capsular polysaccharide is less inflammatory and induces only minor lesions. Alveoli and interlobular septa are filled with edema fluid, and terminal airways and alveoli contain a moderate number of neutrophils (3). Leukotoxin of *P. haemolytica* induces lesions in the lungs of ruminants consisting of greyish consolidated areas with edema of interlobular septa and hemorrhage (22, 23). Microscopically, interlobular septa, pleura, and peribronchial interstitium are expanded because of edema and fibrin deposition. There is thrombosis of lymph vessels, small areas of hemorrhage, and moderate to marked infiltration of intact and degenerated neutrophils and macrophages.

Although the pathogenesis of *P. haemolytica* infection in the lower respiratory tract is complex, examination of individual cell-associated bacterial products has helped identify those that damage tissue and contribute, in part, to the overall lesion seen after experimental pneumonic pasteurellosis. As shown here, these lesions are similar, in part, to the lesions seen in the experimental infection (2, 12).

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