Pulmonary Lesions Induced by *Pasteurella haemolytica* in Neutrophil Sufficient and Neutrophil Deficient Calves

Mike A. Breider, Robert D. Walker, Fred M. Hopkins, T. Wayne Schultz and Terry L. Bowersock*

ABSTRACT

The role of neutrophils in the development of peracute lung lesions of bovine pneumonic pasteurellosis was investigated. Eight calves were divided into two groups of four calves each. Group I was treated with intravenous phosphate-buffered saline and served as the neutrophil sufficient calves. Group II was treated with intravenous hydroxyurea which produced a state of neutropenia. When peripheral blood neutrophil numbers dropped below 300 cells/μL in group II, all calves were challenged with an intrabronchial bolus of *Pasteurella haemolytica* in the log phase of growth. An acute inflammatory process occurred in both groups of calves indicated by a rise in body temperature. While pulmonary lesions occurred in both groups by six hours postinoculation, they varied in pathological characteristics. Pulmonary lesions in the neutrophil sufficient calves consisted of fibrinopurulent alveolitis-bronchiolitis with associated alveolar septal necrosis, interlobular edema, and intravascular thrombi. The neutrophil deficient calves had extensive intra-alveolar edema, interlobular edema, intra-alveolar hemorrhage, atelectasis, and focal areas of alveolar septal necrosis. These results show that *P. haemolytica* can induce severe pulmonary tissue damage through both neutrophil dependent and neutrophil independent mechanisms.

RÉSUMÉ

Cette expérience visait à étudier le rôle des neutrophiles dans le développement des lésions suraiguës de la pasteurellose pulmonaire bovine. Les auteurs utilisèrent à cette fin deux groupes de quatre veaux. Ceux du premier reçurent de l'eau physiologique, par la voie intraveineuse, et servirent de témoins, tandis que ceux du deuxième reçurent, aussi par la voie intraveineuse, de l'hydroxyurée, destinée à provoquer une neutropénie. Lorsque le nombre de neutrophiles des veaux du deuxième groupe eut chuté à moins de 300/μL, ils subirent, tout comme les témoins, une infection de défi bronchique, au moyen d'un bolus de *Pasteurella haemolytica* en phase de croissance logarithmique. Une réaction inflammatoire aiguë se produisit chez les huit veaux, comme le démontra la fièvre qu'ils développèrent. Même si des lésions pulmonaires se produisirent chez les sujets des deux groupes, en l'espace de six heures, elles revêtirent des caractères différents. Les témoins affichèrent une inflammation fibrino-purulente des alvéoles et des bronchioles qui s'accompagnait de nécrose des parois alvéolaires, d'oedème interlobulaire et de thrombose vasculaire. Quant aux veaux chez lesquels on avait provoqué une neutropénie, ils présentaient beaucoup d'oedème alvéolaire et interlobulaire, des hémorragies alvéolaires, de l'atélectasie et une nécrose focale des parois alvéolaires. Ces résultats démontrent que *P. haemolytica* peut provoquer de sévères lésions pulmonaires par l'intermédiaire de mécanismes dépendants ou non des neutrophiles.

INTRODUCTION

Respiratory disease continues to be a serious problem in beef cattle in the United States. The bovine respiratory disease complex (BRDC) is caused by various viral and bacterial agents (1). The most common bacterial agent isolated in acute cases of BRDC is *Pasteurella haemolytica* (serotype 1, biotype A) (2). Many investigators believe viral agents cause damage to the respiratory mucosa, mucociliary clearance apparatus, and pulmonary immune system enabling colonization of the lung by *P. haemolytica* (1).

The peracute lesions of experimental pulmonic pasteurellosis, evident as soon as 6-12 h postinoculation, consist of alveolar wall necrosis, marked neutrophil exudation, intra-alveolar edema, and interlobular edema (3,4). These lesions progress to extensive areas of coagulative necrosis with evidence of vascular thrombosis (5). The specific pathogenic factors that cause these changes have not been well characterized, although *P. haemolytica* endotoxin and leukotoxin have been suggested to be important in providing chemotactic attraction for neutrophils into the lung (4). *Pasteurella haemolytica* leukotoxin has been

*Department of Pathobiology (Breider, Bowersock), Department of Rural Practice (Hopkins) and Department of Animal Science (Schultz), College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37901-1071 and Department of Microbiology, Michigan State University, East Lansing, Michigan (Walker).

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shown to cause marked degeneration and death of both neutrophils and macrophages *in vitro* (6-11). It is postulated that lysis and release of neutrophil lysosomal enzymes play an important role in tissue injury. A previous study demonstrated that neutrophils may have an important role in causing tissue injury following *P. haemolytica* infection (4).

In preliminary studies in our laboratory using hydroxyurea neutrophil depleted calves, we observed that, although pulmonary tissue damage was more pronounced in neutrophil sufficient calves, there were also significant pulmonary lesions in neutrophil deficient calves (12). The purpose of this study was to further investigate the acute pulmonary damage, associated with *P. haemolytica*, in neutrophil deficient and neutrophil sufficient calves. The clinical findings, lung bacteriological culture results, and pulmonary tissue changes following *P. haemolytica* intrabronchial challenge are reported. The role of the neutrophil, neutrophil interaction with different bacterial factors, and neutrophil independent pathogenic factors relating to lung injury are discussed.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Eight cross-bred calves (130-230 kg body weight), obtained from local farms and acclimated to our facility for ten days, were used. Experiments were initially performed on four calves, two animals for each experimental group. Following preliminary results, the experiment was repeated with an additional four animals and the results were added to the initial experimental data. Nasal swabs from all calves were negative for *P. haemolytica*. Serum enzyme immunosorbent assay (ELISA) titers to *P. haemolytica* in all calves were determined as previously described (13). The ELISA titers in all calves used in the experiments were 1:32 or less before treatment. The animals were divided randomly into two equal groups. Group I calves were neutrophil sufficient and group II calves were depleted of neutrophils.

INDUCTION OF NEUTROPENIA

To induce neutropenia, calves in group II were treated with hydroxyurea (Sigma) using a previously described treatment regimen, consisting of daily intravenous injections of 1.0 g hydroxyurea/10 kg body weight for four days (4). Calves in group I were treated with sterile physiological saline intravenously for four days. The animals' hemograms were monitored daily throughout the treatment and posttreatment period. By the fourth day following the last hydroxyurea treatment, all animals in group II had neutrophil numbers less than 300 cells/μL. At this time all calves were challenged with *P. haemolytica.*

PULMONARY CHALLENGE WITH *P. HAEMOLYTICA*

All calves were inoculated with 25 mL of 1.2-2.6 x 10^9 colony-forming units (CFU)/mL viable *P. haemolytica* (isolate 12216 biotype A, serotype 1) in log phase of growth (13). The inoculation method was a modification of a previously described intrabronchial catheterization technique (14). The calves were intubated in a standing position and a modified Foley-type catheter was passed blindly into the bronchus of the right caudal lung lobe. After the catheter cuff was inflated, the bacterial inoculum was injected into the bronchus.

NECROPSY

At 6 h postinoculation all calves were euthanized with intravenous T-61. The lung lobe containing the inoculation site was identified and 1 cm thick serial sections of the inoculated lobe were made to assess the gross lesions. Because the inoculation site was consistently in the right caudal lung lobe (except in calf 79), bacteriological cultures were taken from the right caudal, right middle, right cranial lung lobes, and tracheobronchial lymph nodes. Pulmonary tissue to be cultured was seared, aseptically incised, and the internal parenchyma was swabbed. The swab was subsequently streaked on a BHI plate and estimations of the resultant *P. haemolytica* colony density were made following a 24 h incubation. Tissue sections of the inoculation site were taken in a similar manner in all calves to provide consistent sampling of the lesion center and outer margin. Following fixation in Trumps fixative for 24 h, the tissue was processed for paraffin embedding, sectioned at 6 μm, mounted on glass slides, and stained with hematoxylin and eosin.

RESULTS

CLINICAL AND HEMATOLOGICAL PARAMETERS

At 6 h postinoculation all animals of both groups demonstrated an increased body temperature, with highs ranging from 40.1-41.9°C. There was no difference in body temperature rise between treatment groups. The hematological profiles of both groups of calves are shown in Table I. Following hydroxyurea treatment and before pulmonary challenge, the calves in group II demonstrated a marked decrease of circulating neutrophils and also an approximately 40% decrease of lymphocytes. Six hours following pulmonary challenge, the neutrophil sufficient calves had a moderate increase in circulating neutrophils and a moderate decrease in circulating lymphocytes. The neutrophil deficient calves also had a similar drop in lymphocytes following challenge.

LUNG BACTERIOLOGICAL CULTURES

The results of the postmortem bacteriological cultures are listed in Table II. High bacterial numbers were present in all inoculation sites for both groups except for calves #60 and 1. In three of the neutrophil deficient calves (#51, 56 and 72) and three of the neutrophil sufficient calves (#58, 62 and 70) there were also high numbers of bacteria in at least one additional site in proximity to the inoculation site (i.e. right cranial lobe, right middle lobe, or tracheobronchial lymph node). Positive cultures in calf #70 occurred in the left caudal, left middle, and right cranial lung lobes due to the inoculation site being the left middle lobe.

PATHOLOGICAL FINDINGS

The inoculation site in calves was the right caudal lung lobe, except in calf #70, in which the left middle lobe was predominantly involved. It was difficult to determine the exact size of the inoculation site in both groups due to the acuteness of the lesions and lack of a sharp line of demarcation between involved and uninvolved tissue.

TABLE I. Hematological Profile of Experimental Calves. The Values Represent Means and Standard Deviations of the Four Calves in Each Group

	White Blood Cells	Neutrophils	Lymphocytes
Group I — Neutrophil sufficient			
Pretreatment (PBS)	10,000 ± 2062	3208 ± 1481	4794 ± 2232
Preinoculation[a]	9925 ± 1026	1796 ± 543	7495 ± 362
Postinoculation[b]	9750 ± 722	4922 ± 1362	4112 ± 924
Group II — Neutrophil deficient			
Pretreatment (H-U)[c]	8400 ± 1589	1958 ± 850	6159 ± 744
Preinoculation	4125 ± 653	178 ± 62	3839 ± 747
Postinoculation	1550 ± 683	36 ± 22	1500 ± 683

[a]Immediately before bacterial challenge
[b]Six hours following bacterial challenge
[c]Before hydroxyurea treatment

In the neutrophil sufficient calves (Group I) gross lesions consisted of mild to marked interlobular edema within and adjacent to the inoculation site. The parenchyma in the inoculation site varied from light red to tan. There was increased firmness of the parenchyma in this area and only minimal bronchial exudate. Pulmonary tissue from the inoculation site in these calves contained a severe fibrinopurulent inflammatory process which often effaced normal parenchymal architecture. This process was characterized by extensive interstitial edema with expansion of the interlobular septae due to edema fluid, fibrinous exudate, and moderate numbers of neutrophils (Fig. 1a). The alveolar spaces and bronchiolar lumina were filled with edema fluid, fibrin, neutrophils, and erythrocytes (Fig. 1b). In many areas alveolar structures were indiscernible due to necrosis of the alveolar septae and associated dense accumulations of neutrophils (Fig. 1c). Many neutrophils within alveoli were degenerate as evidenced by streaming cytoplasm, pyknotic nuclei, and cellular lysis. The alveoli also contained lesser numbers of macrophages and eosinophils. In some areas the alveolar spaces were partially lined by cuboidal epithelium suggestive of type II pneumocytes. In one calf (#58), in addition to the aforementioned changes, there was also a moderate degree of alveolar septal fibrosis possibly related to a previous pulmonary disease.

The gross pulmonary lesions of the neutrophil deficient calves (Group II) had a consistent appearance characterized by a central area of marked parenchymal reddening and atelectasis. Surrounding the dark red parenchymal area was a peripheral rim of interlobular edema. The cut edge of the tissue was wet and exuded small amounts of clear red fluid. Extensive microscopic lesions were present in the inoculation site of all calves suggestive of vascular damage. Changes in the tissue were dominated by extensive interlobular and intra-alveolar edema (Fig. 2a). Many areas had retention of alveolar architecture while other areas were atelectatic. There was diffuse marked alveolar edema, hemorrhage, and fibrinous exudate throughout the inoculation sites (Fig. 2b). In two of the calves (#51 and 56) there were prominent bacterial colonies scattered throughout many alveolar spaces, alveolar septae, bronchiolar lumina, interlobular septae, and within interlobular lymphatics (Fig. 2c). In many areas the alveolar septae were expanded due to edema and marked congestion. Occasional areas in the parenchyma contained focal infiltrates of eosinophils with associated alveolar wall necrosis. Occasional small pulmonary arteries had luminal fibrinous thrombi, hypertrophied endothelium, low numbers of mononuclear cells in the tunica media, and perivascular hemorrhage. The mucosal epithelium of most bronchioles had mild to moderate degenerative changes consisting of cellular shrinkage, nuclear pyknosis, and loss of individual epithelium in regional areas leaving a single layer of flattened epithelium.

DISCUSSION

Results from our study show that a significant portion of the tissue damage mediated by *P. haemolytica* is independent of the presence of neutrophils. Even with depletion of peripheral blood neutrophils there was severe pulmonary tissue damage in all neutrophil deficient calves. This tissue damage was evidenced by the grossly visible interlobular edema and extensive microscopic changes charac-

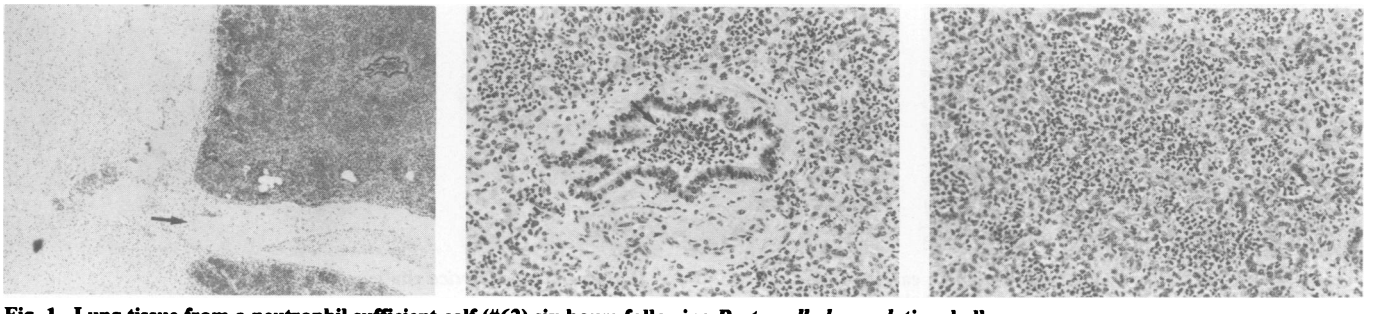

Fig. 1. Lung tissue from a neutrophil sufficient calf (#62) six hours following *Pasteurella haemolytica* challenge.
a. The interlobular septae (arrow) is expanded with edema, fibrinous exudate and neutrophils. H & E. X40.
b. Alveolar spaces and bronchiolar lumina contain neutrophils, bacteria (arrow) and fibrinous exudate. H & E. X200.
c. Effacement of alveolar architecture due to dense neutrophilic infiltrates and alveolar septae necrosis. H & E. X200.

TABLE II. Estimated Density of Bacterial Colonies Per Culture Swab Taken From the Lungs of Necropsied Calvels, Six Hours Following *Pasteurella haemolytica* Challenge of the Right Caudal Lobe

Group	Animal #	RtCa[a]	RtM[b]	RtCr[c]	TBLN[d]
I	58	1000	0	1000	0
	62	100	25	1	0
	70[e]	NA	NA	200	0
	1	0	0	0	0
II	51	1000	100	100	1000
	56	1000	1000	1000	25
	60	1	0	0	0
	72	0	300	1000	0

[a]Right caudal lobe
[b]Right middle lobe
[c]Right cranial lobe
[d]Tracheobronchial lymph node
[e]Left caudal lobe and left middle lobe (inoculation site) had 80 and 100 colonies respectively

terized predominantly by alveolar edema, hemorrhage, atelectasis, and selected alveolar septal necrosis. The rise in body temperature in all neutrophil deficient calves also indicates that an acute inflammatory response did occur in spite of low levels of neutrophils. In a previous study, neutrophil deficient calves developed only mild scattered microscopic lesions, compared to extensive fibrinopurulent inflammation in neutrophil sufficient calves (4). We cannot explain the discrepancy between those results and the results reported in our study. Possible reasons for the differences may be that calves in the previous study were much younger than our calves and also the route of bacterial challenge was different. The intratracheal inoculation of bacteria used in the previous study may not have introduced bacteria in sufficient numbers to a focal site to elicit lesions such as we induced. Differences in results may also relate to the different strain of *P. haemolytica* we used, which was possibly more virulent and therefore resulted in a more severe lesion.

The density of bacterial colonies cultured from infected lungs did not differ significantly between the two treatment groups. Although both calf #1 and #60 had microscopic lesions consistent with pulmonary pasteurellosis, very few or no bacteria were cultured from the pulmonary tissue. This was a surprising finding and may be related to either error in tissue sampling or laboratory culturing techniques.

The extensive alveolar edema, moderate alveolar hemorrhage, endothelial cell hypertrophy, and vasular thrombosis in the neutrophil deficient calves suggests that these changes are related to damage to vascular components. The effects of *P. haemolytica* on bovine pulmonary endothelial cells have not been reported, although it has been demonstrated *in vitro* that *Escherichia coli* endotoxin can induce degenerative changes in bovine pulmonary endothelium (16,17). It is possible that *P. haemolytica* endotoxin or perhaps other *P. haemolytica* toxins may directly damage endothelium. Although the major emphasis of *P. haemolytica* leukotoxin research has been related to its effects on leukocytes, it is possible that leukotoxin may also have some toxic effects on other cells in the lung, specifically pulmonary endothelial cells. We have demonstrated recently in our laboratory that bacteria-free *P. haemolytica* culture supernate (crude leukotoxin) produces a significant endothelial cell cytotoxicity *in vitro* without the presence of neutrophils (Breider MA — unpublished data).

Results from this study do support previous studies demonstrating a peracute inflammatory response to *P. haemolytica* in the bovine lung with considerable tissue damage as early as six hours following challenge (3,4). Although it has been reported that there is a mild neutropenia associated with the acute phase of pasteurellosis (4), neutrophil sufficient calves in this study had a consistent neutrophilic leukocytosis and lymphopenia six hours following infection. These hemogram changes may be related to the acute inflammatory response or may reflect a stress leukogram. The severe acute fibrinopurulent alveolitis and bronchiolitis were similar to that

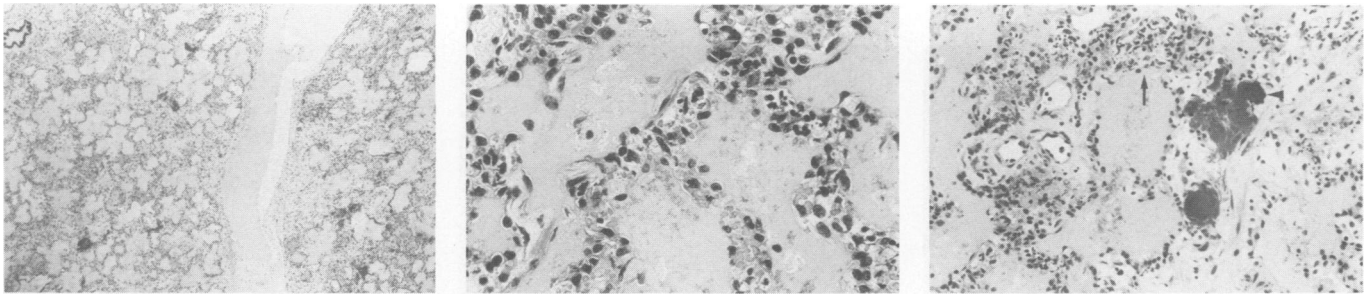

Fig. 2. Lung tissue from a neutrophil deficient calf (#56) six hours following *Pasteurella haemolytica* challenge.
a. Extensive interlobular and intra-alveolar edema. H & E. X40.
b. Close-up of Fig. 2a showing diffuse alveolar edema, intra-alveolar bacteria, and expansion of alveolar septae due to congestion and a mild mononuclear infiltrate. H & E. X400.
c. Prominent interstitial and intra-alveolar bacterial colonies (arrowhead). The interstitium is expanded due to edema. Note the focal alveolar septal necrosis associated with eosinophil aggregates (arrow). H & E. X200.

observed in previous studies characterizing the peracute inflammatory response to *P. haemolytica* (3,4). In addition to the obvious heavy neutrophilic infiltrate into alveolar and bronchiolar spaces, there was also evidence of vascular injury indicated by marked interlobular edema, fibrinous exudate, alveolar hemorrhage, and endothelial swelling. The severe alveolar lesions in the neutrophil sufficient calves demonstrate that the bovine neutrophil is important in mediating some of the damage to the pulmonary parenchyma following *P. haemolytica* infection.

Marked chemotaxis of neutrophils occurs in the lung following *P. haemolytica* inoculation (15). The specific chemotatic factors have not been elucidated in pulmonic pasteurellosis, but activated serum and leukotriene B4, which have significant chemotactic activity for bovine neutrophils, may be important (18). Macrophages, interacting with various bacterial products such as endotoxin, killed *E. coli* cultures, or *E. coli* sterile filtrates, also release chemotactic factors for neutrophils (18). Interaction of alveolar macrophages and peripheral blood neutrophils may be important in pulmonic pasteurellosis. Following neutrophil accumulation in the lung, certain inflammatory mediators and bacterial products may stimulate neutrophils, while other bacterial products depress neutrophil functions. *Pasteurella haemolytica* endotoxin in moderate concentrations has a stimulatory effect on neutrophils, evidenced by increased phagocytosis of *Staphylococcus aureus* (19). Stimulated neutrophils in other similar disease systems in ruminants can cause significant pulmonary disease and cell damage, specifically against endothelium (20). It is feasible that such a situation of stimulated neutrophils mediating tissue damage may exist with bovine pasteurellosis. Another suggested pathogenic mechanism deals with *P. haemolytica* leukotoxin, which has a severely damaging effect on bovine leukocytes, including neutrophils (6-11). Initially the toxin suppresses neutrophil bactericidal functions with subsequent rapid cell degeneration, death, and lysis. Although most data relating to leukotoxin action is based on *in vitro* studies, it is probable that the death and subsequent lysis of the neutrophils with release of lysosomal enzymes damages the alveolar epithelium and endothelium. Studies in rabbits demonstrate that neutrophil lysosomal release may severely damage microvasculature structures leading to microthrombosis and endothelial cell damage (18).

In summary, tissue damage of the bovine lung mediated by *P. haemolytica* appears to be a very acute event with significant lesions developing within six hours postinoculation. The precise mechanisms of tissue damage are not yet evident but these studies show that both neutrophil dependent and neutrophil independent mechanisms are important in causing pulmonary damage. Additional work is needed to further define which inflammatory mechanisms are important in mediating tissue damage and what bacterial factors initiate these mechanisms. It is only by the definition of these pathogenic mechanisms and bacterial factors that effective prophylactic and treatment regimens can be developed to control this persistent disease problem in cattle.

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