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The Role of Leukocytes in the Pathogenesis of Fibrin Deposition in Bovine Acute Lung Injury

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The peculiarly fibrinous nature of bovine acute lung injury due to infection with* Pasteurella haemolytica *A1 suggests an imbalance between leukocyte-directed procoagulant and profibrinolytic influences in the inflamed bovine lung. Calves with experimental pneumonia produced by intratracheal inoculation with* P. haemolytica *A1 developed acute locally extensive cranioventral fibrinopurulent bronchopneumonia. Pulmonary alveolar macrophages (PAM) recovered by segmental lavage from affected lung lobes were 30 times more procoagulant than PAM obtained from unaffected lung lobes and 37-fold more procoagulant than PAM from control calf lungs. Unlike the enhancement of procoagulant activity, profibrinolytic activity (plasminogen activator amidolysis) of total lung leukocytes (PAM and plasminogen activator neutrophils [PMN]) was decreased 23 times in cells obtained from affected lung lobes and also was decreased four times in cells obtained from unaffected lobes of infected animals. This marked imbalance in cellular procoagulant and fibrinolytic activity probably contributes significantly to enhanced fibrin deposition and retarded fibrin removal. In addition, PAM from inflamed lungs were strongly positive for bovine tissue factor antigen as demonstrated by immunocytochemistry. Intensely tissue factor-positive PAM enmeshed in fibrinocellular exudates and positive alveolar walls were situated such that they were likely to have, in concert, initiated extrinsic activation of coagulation in the acutely inflamed lung. These data collectively suggest that enhanced PAM-directed procoagulant activity and diminished PAM- and PMN-directed profibrinolytic activity represent important modifications of local leukocyte function in bovine acute lung injury that are central to the pathogenesis of lesion development with extensive fibrin deposition and retarded fibrin removal. (Am J Pathol 1991, 138:1191–1198)

The peculiarly fibrinous nature of acute bovine bronchopneumonia due to *Pasteurella haemolytica*[1,2] suggests an imbalance between procoagulant and profibrinolytic activities in the lung that impedes normal fibrin turnover. Increased procoagulant activity in bronchoalveolar fluid or pulmonary alveolar macrophages (PAM) and/or decreased profibrinolytic activity are believed to predispose the alveolus to fibrin deposition in many natural[3–6] and experimental pulmonary diseases.[7–11] The deleterious consequences of fibrin deposition are well described[12–14] and are consistent with the fibrosis observed in chronic lesions of bovine fibrinous bronchopneumonia.[15,16] Alveolar epithelial injury,[17] the deposition of a fibrin–fibronectin matrix,[12,18] and trophic factors elaborated by macrophages[19–27] probably combine to promote fibroblast attachment, chemotaxis, spreading,[28] and collagen production in the healing lung.[29–31]

Promotion of extrinsic coagulation and of plasminogen activation as likely sources of lung activity[32–35] was measured in leukocytes recovered from normal lung and from unaffected and diseased lobes of infected animals to evaluate fibrin turnover in pneumonic calves. Normal and diseased lung and pulmonary leukocyte preparations were examined immunocytochemically for the presence and distribution of tissue factor because recent evidence suggests that alveolar epithelium and septae, as well as PAM, potentially may contribute to fibrin deposition in the pulmonary alveolus.[36]

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Materials and Methods

Pneumonic Calf Model

Eight 1-week-old male Holstein calves purchased from the Cornell University Teaching and Research Center[37] were made pneumonic by the intratracheal inoculation of 10^9 logarithmic growth-phase *Pasteurella haemolytica* A1 organisms according to the method of Friend.[2] Eight normal animals were used to obtain normal data. Bacteria were inoculated into bovine brain heart infusion broth (BHI, Gibco, Grand Island, NY) and grown for 6 hours before being pelleted at 3000*g* for 15 minutes, washed in saline, and resuspended in 50% BHI/sterile saline at 10^8 cells/ml. Twenty milliliters of bacterial suspension were inoculated intratracheally at the point of inspiration in laterally recumbent, xylazine (Butler, Columbus, OH) tranquillized calves. Bacterial concentration subsequently was confirmed with the colony-forming unit assay. Mean inoculum was 10^9 cells. After 22 hours calves were killed with 2 to 4 sodium thiamylal (Boehringer Ingelheim, Indianapolis, IN) injected intravenously. All affected lungs were cultured and essentially pure *P. haemolytica* was isolated in each case.

Bronchopulmonary Lavage

The deeply fissured nature of bovine lungs enabled us to separate and detach the lobes. Individual unaffected lobes were lavaged five times intrabronchially with a solution of 20% acid citrate dextrose (ACD)/sterile saline. For affected lobes similarly separated, the pleural surface was cross-hatched to a depth of 3 to 5 mm with a sterile scalpel, and 20% ACD/saline was introduced intrabronchially with the lobe suspended over a beaker. This alternate procedure served to increase cell yield without increasing recovered interstitial cells, altering differential determinations or measured activities compared with exbronchial recovery as determined in preliminary experiments. The wash procedure was repeated several times. Bronchoalveolar fluid was filtered rapidly through sterile cotton gauze and placed on ice.

Cell Isolation

Bronchoalveolar fluid from pneumonic lobes was pelleted at 200*g* for 15 minutes.[37] Hypotonic flash lysis of pelleted cells was performed routinely as previously described for the purification of bovine blood neutrophils[38] to remove red blood cell contamination. Directly after lavage and after washing cytocentrifuge preparations were obtained and stained with Wright-Giemsa to determine the differential cell count.

Procoagulant Assay

The one-stage procoagulant assay performed was a modification of the prothrombin time assay.[39] Cells collected as described above were pelleted by centrifugation (600*g*, 15 minutes), the supernatant carefully removed by pipette to prevent disturbing the cell pellet, and the pellet resuspended in 0.5 ml 30 mmol/l (millimolar) $CaCl_2$ saline. Samples then were prewarmed to 37°C. Pooled platelet-poor bovine plasma (PPP) was preincubated (3 minutes at 37°C) in fibrometer cups. Modified prothrombin time was measured as the time required to clot 0.1 ml PPP after the addition of 0.2 ml cells suspended in 30 mmol/l $CaCl_2$ saline. Clotting times were measured in duplicate on a fibrometer (BBL, Becton Dickinson, Rutherford, NJ). Log/log standard curves of rabbit thromboplastin (Simplastin, Sigma, St. Louis, MO) with pooled bovine PPP were used to interpolate equivalent time/dilution values. Rabbit and bovine tissue factor are unequally active in bovine plasma[40]; however Simplastin (rabbit brain thromboplastin) provided a convenient and consistent standard for comparison. Procoagulant activity units used in text represent tissue factor equivalent concentrations, being the reciprocal of this concentration × 1000. The activity measured in this assay was 100% inhibitable by the rabbit anti-bovine tissue factor antibody used later in immunocytochemistry at levels greater than 10 μg/ml (data not shown). Bovine peripheral blood neutrophils enriched as previously described, [38] either freshly isolated or after stimulation with either 5 μg/ml 0111:B4 lipopolysaccharide (LPS), or 20 ng/ml phorbol myristate acetate for up to 8 hours in Hank's Balanced Salt Solution (HBSS; Gibco) with 0.1% bovine serum albumin (BSA) failed to clot the pooled bovine plasma used in this assay (ie, no clot formed by 600 seconds).

Plasminogen Activator Assay

The plasminogen activator activity of frozen samples of cell lysates (0.25% Triton X-100) was determined using an amidolytic assay that measured the cleavage of substrate S-2444 (Kabi, Helena Laboratories, Beaumont, TX) as described.[41] This substrate is highly specific for urokinase-type plasminogen activator. While amidolytic activity does not necessarily parallel the fibrinolytic activity for different urokinase species,[41] samples of bovine PAM, including cell lysates and conditioned media as well as bronchoalveolar lavage fluid from normal and pneumonic animals, were shown to produce directly proportional ac-

tivities in the plasminogen-dependent multiwell fibrin plate fibrinolytic assay of Moroz[42] (data not shown).

Immunocytochemistry

Lung samples from normal and affected lobes were frozen in optimal cutting temperature compound (Miles Scientific, Naperville, IL) in isopentane supercooled in liquid nitrogen. Cryostat sections were cut at 4 μm and used unfixed. Cytospin preparations of leukocytes from pneumonic and diseased lobes were frozen at −70°C and used unfixed. Immunocytochemistry was performed using rabbit antibovine tissue factor IgG (provided by Dr. Ronald Bach, Mt. Sinai Medical Center, New York, NY) with the modified immunoperoxidase method, and Zymed kits for endogenous avidin-biotin blocking and immunoperoxidase staining (Zymed Laboratories, San Francisco, CA) using 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, MO) as the chromogen.[43] Following immunoperoxidase staining, sections were counterstained with Gill's hematoxylin for 1 minute. Paraffin-embedded, hematoxylin and eosin-stained 5-μm sections were contrasted to immunocytochemically stained sections. Negative controls were used throughout (rabbit serum antibody) to ascertain staining specificity. The anti-bovine tissue factor antibody (more than 10 μg/ml) was shown to inhibit completely expression of maximally stimulated PAM procoagulant activity (cultured in HBSS, 0.1% BSA, 5 μg/ml LPS for 8 hours at 37°C) in a one-stage procoagulant assay.

Statistics

Independent samples were compared using the nonparametric Wilcoxon signed rank test.

Results

Lesion Distribution and Cell Recovery

The diaphragmatic lobes diagonally opposite affected anterior lobes were chosen as internal control lobes (Figure 2A). These lobes were essentially normal by both gross and histologic evaluation; some focal collections of neutrophilic infiltration were observed. Few or no bacteria were cultured from these lobes while pure, dense cultures of *P. haemolytica* A1 grew from all cultured affected lobes. Cells recovered from pneumonic calves included neutrophils, macrophages, some lymphocytes, and rare epithelial cells. The proportion of PAMs decreased from 95% ± 5.6% in normal calves to 83.6% ± 17.1% in the control lobes of affected calves to 16.6% ± 8.9% in consolidated lobes of affected calves (Figure 1A). These percentages were significantly different comparisons of normal to diseased ($P < 0.02$) and control to diseased ($P < 0.02$) lung lobes.

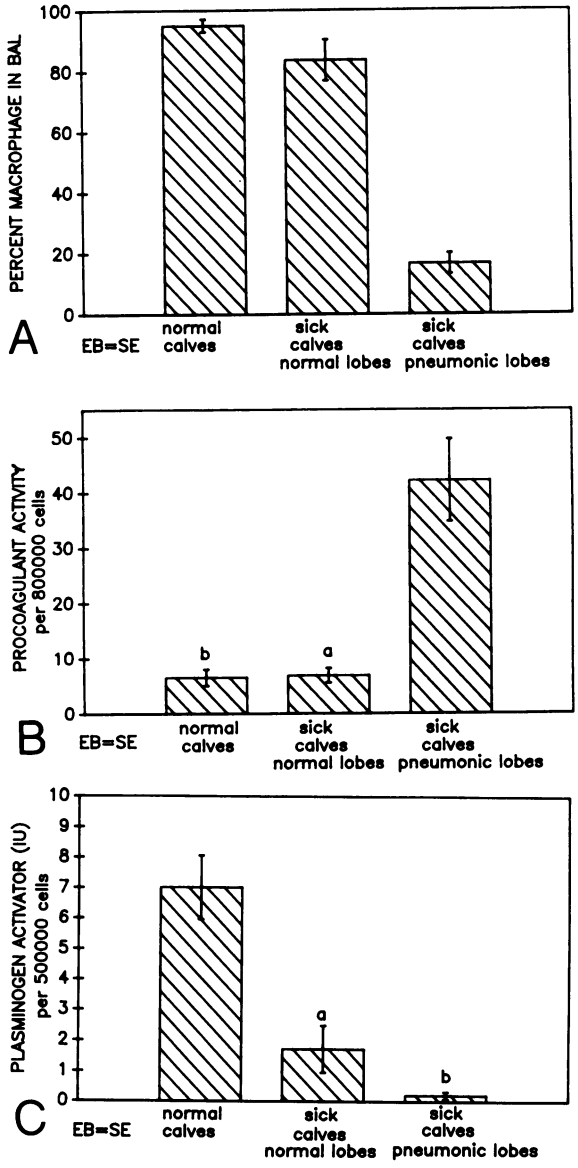

Figure 1. A: *Proportions of bovine pulmonary alveolar macrophages (PAM) recovered in bronchoalveolar lavage fluid. Values represent the mean percentage of PAM counted on Wright-Giemsa–stained cytospin preparations.* B: *Procoagulant activity of bovine pulmonary total leukocytes from normal calves, and from normal and diseased lobes of pneumonic calves. Values represent the mean procoagulant activity of 8 × 10^5 cells measured as freshly recovered from lung. For the statistical difference, a, P < 0.02, and for b, P < 0.02. compared to pneumonic lobes.* C: *Plasminogen activator amidolytic activity of bovine pulmonary total leukocytes from normal calves, and from normal and diseased lobes of pneumonic calves. Values represent the mean plasminogen activator activity of 5 × 10^5 cells measured by the amidolytic assay as freshly recovered from lung and lysed in 0.25% Triton X-100. For the statistical difference, a, P < 0.05, for b, P < 0.03 (both compared to normal calves), and for a/b, P > 0.1. Error bars are calculated as standard error margins (EB = SE).*

Procoagulant Activity

Procoagulant activity was measured on a per-cell basis (PAM + PMN) at 6.5 ± 1.5 units and 6.9 ± 1.3 units for cells obtained from normal and control lobes, respectively, and 42.2 ± 7.5 units ($P < 0.02$) for cells obtained from affected lobes (Figure 1B). Therefore a sixfold increase in procoagulant activity was observed in procoagulant activity for cells collected from pneumonic lobes. Because bovine neutrophils (PMN) do not contribute to procoagulant activity[44] (see Materials and Methods), these figures were corrected for PAM concentration. PAM-directed procoagulant activity in affected lung lobes was thus increased 37 times over that obtained with PAM from normal lungs, and increased 30 times over that obtained with PAM from unaffected lobes of pneumonic calves.

Plasminogen Activator Amidolytic Activity

Because both bovine PAM and PMN produce plasminogen activator, amidolytic activities measured with the Kabi urokinase substrate S-2444 were compared on a per cell (5×10^5 cells) basis (Figure 1C). Fibrinolytic activity of cells from normal lungs was 7.01 ± 0.82 IU. Fibrinolytic activity of cells obtained from unaffected lobes of pneumonic calves was decreased four times (1.72 ± 0.77 IU; $P < 0.05$), while activity of cells from affected lobes of pneumonic calves was decreased 23 times (0.30 ± 0.12 IU, $P < 0.03$).

Gross and Histologic Lesions

Inoculation of 10^9 *P. haemolytica* A1 intratracheally calves in lateral recumbency produced a relatively severe, but localized unilateral, cranioventral bronchopneumonia (Figure 2A). Diagonally opposite apical and diaphragmatic lobes were almost completely spared and were without significant histologic evidence of inflammation or edema. The intermediate lobe often was affected contralateral to the more severely affected side (Figure 2A). Histologically a typically severe fibrinopurulent alveolitis, with adjacent bronchiolitis, bronchitis, and pleuritis was observed (Figure 2B). Fibrin distended subpleural and interlobular septae as well as peribronchial lymphatics. Many PAMs were seen embedded within alveolar fibrin deposits (Figure 2B, arrow heads).

Immunohistochemistry

Immunocytochemical identification of bovine tissue factor in control lobes revealed diffuse and moderately intense staining of the alveolar wall, including alveolar epithelial cells (Figure 3A, B). Perivascular connective tissue was weakly positive and vascular smooth muscle was moderately positive. Vascular and lymphatic endothelia were uniformly negative, as was bronchiolar and bronchial epithelia. Alveolar macrophages varied in staining intensity in normal lobes from negative to weakly positive (Figure 3B).

In pneumonic lung, alveolar septae and epithelial cells stained moderately intensely, while tracts of neutrophils were negative (Figure 3C, D). Lying within intra-alveolar fibrinocellular exudate and fibrin deposits, many intensely positive PAM were present (Figure 3D), resembling by size, frequency, and location those mononuclear cells observed enmeshed in fibrin in hematoxylin and eosin-stained lung sections (Figure 2B). These PAMs were much more intensely stained than those in normal lung. The designation of such positive cells in pneumonic lung as macrophages was confirmed by the presence of similar cells with oval to amoeboid nuclei and heavily stained

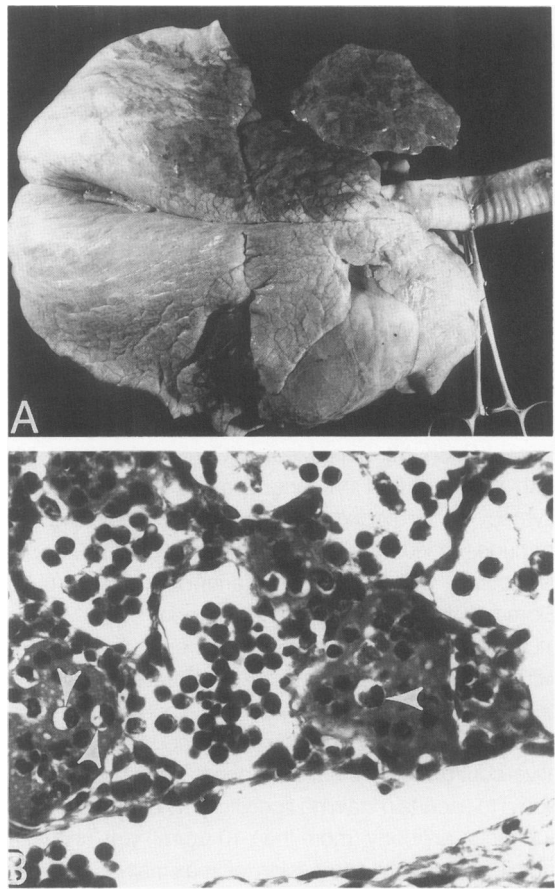

Figure 2. A: *Gross specimen of experimentally induced bovine pneumonic pasteurellosis. Note upper (right side) anteroventral consolidation, and lower (middle) intermediate lobe consolidation. Lower diaphragmatic and apical lobes are grossly normal lobes.* **B:** *Hematoxylin and eosin-stained section of paraffin-embedded pneumonic lung demonstrating macrophages embedded within alveolar fibrin deposits (arrow heads) (original magnification, ×1690).*

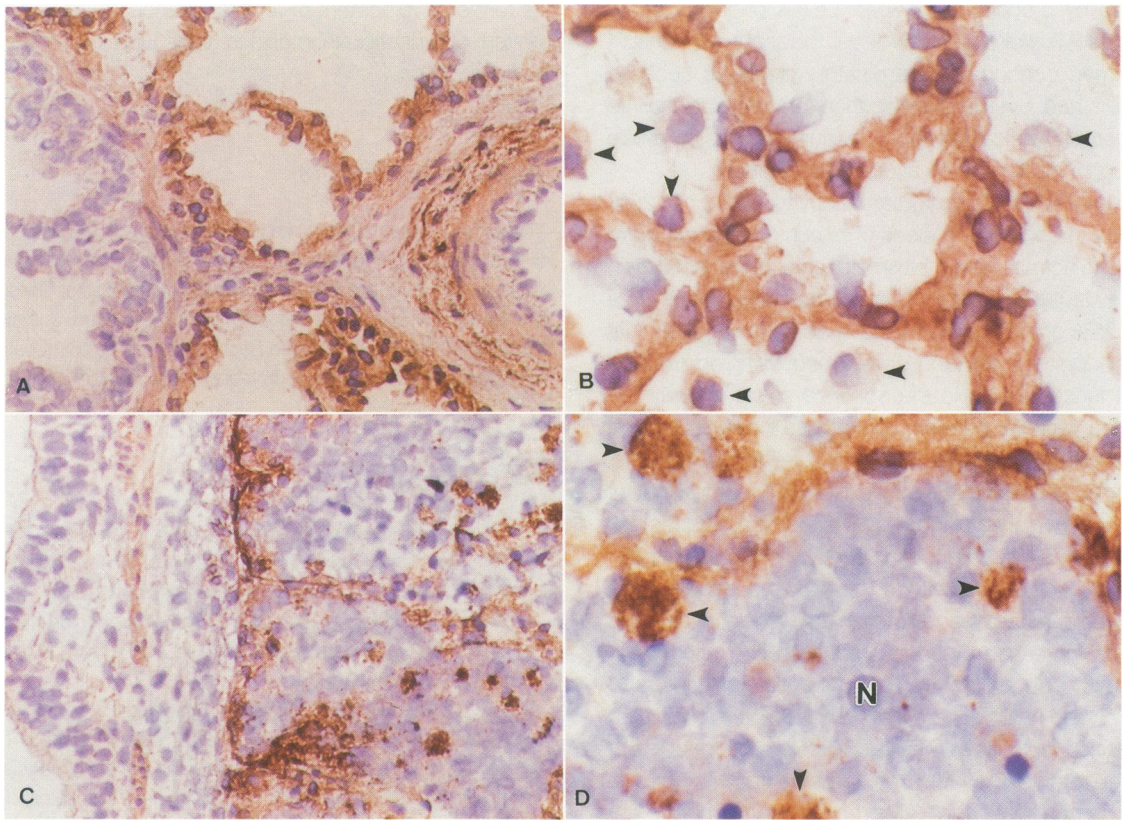

Figure 3. A–D: *Immunoperoxidase stains for bovine tissue factor (rabbit antibovine tissue factor IgG), counterstained with Gill's hematoxylin. Magnifications are listed in parentheses.* **A:** *Normal lung with positive staining restricted to alveolar epithelial cells and alveolar septae, while bronchial epithelium and vascular endothelium are negative (original magnification, ×640)* **B:** *Alveolar macrophages (PAM, arrow heads) in normal lung are variably and weakly positive (original magnification, ×1600)* **C:** *Pneumonic lung strongly demonstrating alveolar wall and alveolar leukocyte positivity, bronchial epithelium is negative (original magnification, ×640)* **D:** *Consolidated pneumonic lung demonstrating strongly positive alveolar leukocytes, probably alveolar macrophages (arrow heads), while fibrinosuppurative exudate (N) is negative (original magnification, ×1600).*

granular cytoplasm present in immunostained cytospin preparations of leukocytes recovered from pneumonic lung (not shown).

Discussion

Naturally occurring acute lung injury in calves due to *P. haemolytica* is a prototypic fibrinous bronchopneumonia, a morphologic manifestation of peculiar severity in the bovine species. In our experimentally reproduced disease, a marked imbalance in procoagulant and profibrinolytic activities between grossly normal and pneumonic lung lobes in pneumonic animals and between pneumonic lung lobes and those of normal animals was observed. While PAM procoagulant activity was increased 37 times in pneumonic lungs, total leukocyte plasminogen activator amidolytic activity was diminished 23 times. Similar imbalances were observed and implicated in alveolar fibrin deposition in interstitial pulmonary diseases in humans[6,45] and after experimental lung injury in sheep,[10] baboons,[46] and rabbits.[8]

The magnitude of the abnormal *in vivo* functional modifications observed was greater than those experimentally induced in populations of PAM and neutrophils (PMN) *in vitro*,[44] although endotoxin- and phorbol ester-stimulated procoagulant activity levels in PAM may approach those attained *in vivo*. Alveolar epithelial cells and septae clearly must contribute to the deposition of alveolar fibrin based on the intense tissue factor antigen staining observed in both control and diseased lung. Whether such staining is surface associated, and thus able to promote fibrin formation is unclear; however it was documented that saturating amounts of tissue factor–factor VII complexes are present on pulmonary alveolar surfaces.[34] The observation of many strongly tissue factor antigen-positive macrophages enmeshed in fibrinocellular exudates directly implicates their role in extravascular alveolar initiation of coagulation.

The flooding of alveoli with α-2 antiplasmin[10,46,47] and the acute-phase reactants plasminogen activator inhibitor type 1,[46,48] a bovine endothelial product stimulated by endotoxin[49] and fibrinogen,[50–52] both at elevated concentrations, would likely facilitate fibrin deposition in-

duced by procoagulant PAMs. Levels of fibrinogen as high as 2.3 g/dl were recorded in bovine pneumonia.[52] Common concurrent inflammatory diseases such as mastitis and juvenile diarrhea therefore are also likely to exacerbate clinical severity of pneumonic pasteurellosis through induction of the acute-phase reaction.

While PAM procoagulant activity was only 20% greater in control lung lobes of pneumonic calves compared to normal calves, plasminogen activator amidolytic activity levels in normal lobes of pneumonic calves were only 25% of those in normal animals. This suggests that local or systemic influences were modulating control lobe PAM function even in the absence of significantly enhanced vascular permeability in those lobes, as judged histologically. Greater lability of modulation for PAM plasminogen activation also may exist than for procoagulant activity *in vivo*. Plasminogen activator inhibitor elaboration may have contributed to the observed reduction of plasminogen activator activity.[53] The effects of endotoxemia on alveolar macrophage function are well described and include increased cytokine production,[54,55] altered adherence, phagocytic, and chemotactic functions, and augmented H_2O^2 generation.[54] The effects of circulating endotoxin potentially may account for differences observed between PAMs from grossly normal lobes in pneumonic calves and PAMs recovered from normal animals.

A role for bovine pulmonary intravascular macrophages in the pathogenesis of bovine acute lung injury is probable, particularly in the induction of the vascular thrombosis and coagulation necrosis commonly observed in both naturally occurring and experimental disease,[1,2] and possibly also in the generation of cytokines that effect alveolar populations of leukocytes and recruitment of neutrophils to the lung.[56–59] The experimental observations that pulmonary intravascular macrophages in calves clear 93% of circulating endotoxin[60] and that bovine macrophages are exquisitely sensitive to endotoxin induction of procoagulant activity[45] probably combine to create a pulmonary capillary bed lined by highly procoagulant macrophages.

The appearance of viable macrophages within fibrinous exudate, together with their ability to promote coagulation and their potent long-term fibrinolytic potential clearly denote their importance in regulating bovine pulmonary turnover of fibrin in inflammatory states. While the contribution of the neutrophil to pulmonary fibrin turnover is uncertain, their ability to release urokinase plasminogen activator in response to stimulation may imply a role in the removal of alveolar fibrin.[61]

Complex interactions between humoral and leukocytic procoagulant, anticoagulant, profibrinolytic, and antifibrinolytic activities thus combine with the markedly procoagulant surfaces of the bovine lung to predispose cattle to the development of a richly fibrinous morphologic pattern of bronchopneumonia in acute lung injury due to *P. haemolytica*.

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