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Abstract

Objectives—To determine effects of selectin inhibitor TBC1269 on neutrophil infiltration, and neutrophilassociated injury during pneumonia induced by *Mannheimia haemolytica* and concentration of antimicrobial anionic peptide (AAP) in bronchoalveolar lavage fluid (BALF) as well as antimicrobial activity of BALF from healthy (control) neonatal calves, neonatal calves with *M haemolytica*-induced pneumonia, neonatal calves with prior treatment with TBC1269, and adult cattle.

Animals—Eighteen 1- to 3-day-old calves and 9 adult cattle.

Procedure—Calves were inoculated with *M haemolytica* or pyrogen-free saline (0.14*M* NaCl) solution into the right cranial lung lobe, and BALF was collected 2 or 6 hours after inoculation. Thirty minutes before and 2 hours after inoculation, 4 calves received TBC1269. The BALF collected from 9 adult cattle was used for comparison of BALF AAP concentration and antimicrobial activity. Protein concentration and neutrophil differential percentage and degeneration in BALF were determined. An ELISA and killing assay were used to determine BALF AAP concentration and antimicrobial activity, respectively.

Results—Total protein concentration was significantly decreased in BALF from calves receiving TBC1269. Similar concentrations of AAP were detected in BALF from all calves, which were 3-fold higher than those in BALF from adult cattle. However, BALF from neonates had little or no anti-*M haemolytica* activity.

Conclusions and Clinical Relevance—These results suggest that TBC1269 decreases pulmonary tissue injury in neonatal calves infected with *M haemolytica*. Although AAP is detectable in neonatal BALF at 3 times the concentration detected in adult BALF, neonatal BALF lacks antimicrobial activity for *M haemolytica*.

Disciplines
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*M*annheimia haemolytica is an important pathogen of ruminants, and it colonizes the tonsils and pharyngeal area of the respiratory tract of cattle.[1,2] During transport or other stresses, bacteria increase in number[3,4] and induce an acute inflammatory response in the lungs characterized by dense infiltrates of neutrophils into alveolar spaces and tissue damage.[5-7] Release of substances such as elastase, myeloperoxidase, and oxidative free radicals from neutrophils has been associated with tissue damage[8-10] and plays a role in the pathogenesis of pneumonia attributable to *M haemolytica*.[11,12] Additional evidence of the importance of neutrophils in *M haemolytica*-induced pneumonia was indicated in the reduction of tissue damage and vascular leakage of protein by depletion of neutrophils.[13-16] Neutrophil-mediated damage to the respiratory tract removes the protective barrier provided by endothelial cells and mucosal epithelium,[11-14,16] allowing access of bacteria and inflammatory mediators to the exposed capillaries, which results in reduction of gaseous exchange. Such damage also may impair innate host defense mechanisms, including mucociliary clearance and production of **antimicrobial peptide (AMP)**.

In response to an infectious agent within alveolar, bronchiolar, or bronchial spaces, neutrophils infiltrate into these areas by moving from the bloodstream, through the endothelium, and through the perivascular tissue and pulmonary parenchyma until they reach the pulmonary spaces. Adhesion molecules expressed by neutrophils and endothelial cells mediate neutrophil extravasation. Selectin adhesion molecules mediate the initial relatively unstable tethering step of neutrophil adherence to the vasculature by binding sialyl Lewis[x] and related oligosaccharide receptors. There are 3 types of selectins: L-selectin, which is detectable on neutrophils and other leukocytes and binds ligands on endothelial cells,[17] and E- and P-selectin, which are expressed primarily by endothelial cells and bind ligands on neutrophils.[18-20] This initial selectin-ligand binding initiates expression of β_2-integrins on the surface of neutrophils,[21] which mediate a more stable adherence to an endothelial **intracellular adhesion molecule (ICAM)** in preparation for movement through the endothelium and into tissues. Inhibition of the initial selectin-mediated tethering of neutrophils to endothelium may play a role in reduction of neutrophil infiltration into the lungs during acute *M haemolytica*-induced pneumonia.

Investigators have used TBC1269, a nonoligosaccharide selectin antagonist, to competitively inhibit

binding of sialyl Lewis[x] to selectins in vitro.[22-25] This mimetic has been used in vivo in clinical studies for use in the treatment of humans with allergic asthma. It is effective for preventing neutrophil-mediated reperfusion injury in rat liver[23] and in reducing the initial cellular response to pulmonary antigen in a sheep model of allergic bronchoconstriction.[25] Inhibition of selectin-mediated neutrophil infiltration may reduce neutrophil-mediated pulmonary damage, thereby maintaining the epithelial barrier and its associated defenses.

Antimicrobial peptides are substances that have antibacterial, antifungal, and antiviral properties in vitro and in vivo.[26,27] Several families of AMP are produced in the lungs of cattle[28-30] and are believed to have an important role in the innate immune system for protection of the lungs from bacterial invaders. One AMP in cattle is **antimicrobial anionic peptide (AAP)**. This peptide is smaller than most AMP, and its anionic properties differ from most AMP, which are cationic.[31] It is composed of consecutive aspartates and requires zinc as a cofactor.[31] Antimicrobial anionic peptide initially was isolated from ovine surfactant. It also is evident in human lung tissue and is detectable in high amounts in **bronchoalveolar lavage fluid (BALF)** collected from humans and sheep.[31,32] Antimicrobial anionic peptide is bactericidal against *M haemolytica* as well as other bacteria at a minimum inhibitory concentration comparable to that of other AMP such as α- and β-defensins.[31] Antimicrobial anionic peptide is believed to be a cleaved fragment secondary to a larger proprotein produced in the lungs.[31] In contrast to AAP, some bovine epithelial β-defensins such as tracheal antimicrobial peptide and lingual antimicrobial peptide usually are not found in mucus and serous respiratory secretions. Instead, those compounds are found within epithelial cells and are locally secreted following their induction.[30,33,34] To our knowledge, the concentration of AAP in BALF of neonatal calves has not been assessed.

The objectives of the study reported here were to test the extent to which inhibition of neutrophil adherence with TBC1269 would affect neutrophil infiltration into the BALF of neonatal calves inoculated with *M haemolytica* as well as neutrophil-associated lung injury and to discern whether AAP is detectable in BALF of neonatal calves.

Materials and Methods

Animals—Eighteen colostrum-deprived male neonatal (1 to 3 days old) Holstein calves were purchased from a university dairy farm and transported to laboratory animal facilities at our institution. They were maintained in accordance with guidelines approved by an institutional animal care and use committee. Calves were monitored daily for general health (appetite, attitude, respiratory effort and sounds, and nasal discharge). Calves were excluded from the study if they were febrile, coughed excessively, had abnormal hematologic and serologic values, or had other signs of illness.

Procedure—One to 3 days after arrival, blood was collected from a jugular vein, and calves were assigned to groups. Calves were anesthetized, and 12 were inoculated with *M haemolytica* (1×10^8 **colony forming units [CFU]**/ml) in a 5-ml injection, as described elsewhere[7,35,36]), whereas 6 were inoculated with 5 ml of pyrogen-free saline (0.14*M* NaCl) solution. Inoculations were performed into the

bronchus of the right cranial lung lobe, using fiberoptic bronchoscopy. One group of calves inoculated with *M haemolytica* received TBC1269 (25 mg/kg of body weight, IV, 30 minutes before and 2 hours after bacterial inoculation).

Calves were euthanatized 2 or 6 hours after inoculation. Calves were euthanatized by IV injection of an overdose of sodium pentobarbital immediately prior to necropsy and collection of BALF. To avoid hyperstatic congestion and potential vascular leakage in postmortem specimens, BALF was collected from each calf within 5 to 15 minutes after it was euthanatized. Each group was comprised as follows: saline-inoculated (control) calves euthanatized 2 (n = 3) and 6 (3) hours after inoculation; *M haemolytica*-inoculated calves euthanatized 2 (4) and 6 (4) hours after inoculation; and *M haemolytica*-inoculated TBC1269-treated calves (4) euthanatized 6 hours after inoculation.

***Mannheimia haemolytica* inoculum**—*Mannheimia haemolytica* L101 were grown overnight on blood agar, transferred to tryptose broth, and incubated for 1 to 3 hours at 37 C on a magnetic stirrer to achieve a culture containing approximately 1×10^8 CFU/ml. The organisms were pelleted, using centrifugation (5,900 $\times$ g for 5 minutes at 4 C), and resuspended in 5 ml of pyrogen-free saline solution. An aliquot of saline solution was adjusted to a transmittance value of 78% at a setting of 600 nm in a spectrophotometer[a] by aseptically adding the bacterial suspension. One milliliter of the inoculum was removed from the adjusted suspension and serially diluted (1- to 10-fold) in saline solution. Then, 0.1 ml of each dilution was spread onto agar plates containing trypticase soy agar with 5% defibrinated sheep blood. Plates were incubated overnight, and colonies then were counted. The original solution routinely contained 1.3 to 2.5 $\times 10^8$ CFU/ml.

Production of sialyl Lewis[x] nonoligosaccharide mimetic (TBC1269)—The compound TBC1269, a synthetic sialyl Lewis[x] nonoligosaccharide mimetic antagonist (1,6-Bis[3-(3-carboxymethylphenyl)-4-(2-α-D-mannopyranosyl-oxy)phenyl]hexane) was produced and provided in a powder form.[22,b] The compound TBC1269 inhibits binding (by 50%) of human L-, E-, and P-selectins at concentrations of 87, 105, and 17 μ*M*, respectively.[25] This compound does not have cytotoxic effects on neutrophils as documented by safety and efficacy studies in rats and dogs.[23] The required dose was prepared in sterile pyrogen-free saline solution (pH 7.4).

Collection of samples—Blood samples were collected from each calf before inoculation and at time of euthanasia for CBC and fibrinogen determination. Because calves were colostrum-deprived and, therefore, acutely susceptible to infections, blood values obtained before inoculation were assessed for indications of systemic disease. Only calves with baseline results of CBC that were within reference ranges were used for the study. Hematologic variables were compared between samples obtained before and after inoculation for all calves used in the study.

In all calves, BALF was collected from the right cranial lung lobe during necropsy. This lobe was dissected from the remainder of the lungs, and 50 ml of pyrogen-free saline solution was delivered into the main bronchus via a nozzle-tipped syringe. This fluid immediately was gently aspirated back into the syringe, and the volume of fluid was measured. For all samples obtained from neonates, a portion of BALF was reserved for cytologic assessment, and a portion was centrifuged to remove cellular debris. Supernatant was retained and frozen (–70 C) until assessed, using Bradford analysis to determine protein concentration, an ELISA to determine concentration of AAP, and a killing assay to determine antimicrobial activity. In addition, BALF was collected from 9

healthy adult (> 1 year old) cattle that did not have a previous history or clinical signs of respiratory tract disease. In these cattle, BALF was collected from the right tracheal bronchus during an anesthetic episode, similar to the procedure described in sheep.[37] For all samples obtained from adult cattle, BALF was centrifuged to remove cellular debris. Supernatant was retained and frozen (–70 C) until assessed, using an ELISA to determine concentration of AAP and a killing assay to determine antimicrobial activity.

Cytologic assessment of BALF—Bronchoalveolar lavage fluid from each neonatal calf was assessed to determine amount collected, degree of transparency, and color. A nucleated cell count was performed, using an automated hematology analyzer,[c] and the value was rounded to the nearest tenth. Fluid protein content was estimated, using a refractometer,[d] and pH was determined. A differential cell count and cytologic examination were performed on each sample (direct smear and centrifuge preparations) after use of Wright stain, and neutrophil degeneration was classified and graded (0, nondegenerate; 1, slightly degenerate; 2, moderately degenerate; 3, degenerate; 4, severely degenerate). Neutrophil morphology was classified by use of a predetermined scoring system: 0, nondegenerate neutrophils that contained clumped basophilic chromatin and appeared similar to neutrophils in other blood samples with only a slight allowance for nuclear swelling attributable to washing; 1, slightly degenerate neutrophils that had intact but mildly dispersed chromatin with mild nuclear swelling that altered the tinctorial property from dark basophilic to dark eosinophilic; 2, moderately degenerate neutrophils that contained nuclei with a dispersed eosinophilic chromatin pattern, and nuclei were swollen to twice normal width but still retained a lobulated pattern; 3, degenerate neutrophils that had a minimal chromatin pattern with a smooth nuclear content, light eosinophilic staining, and a nucleus swollen to fill three fourths of the cell with loss of a lobulated pattern; 4, severely degenerate neutrophils that often had a ruptured cell membrane and a smooth light eosinophilic nucleus that was rounded with loss of the lobular pattern and lack of chromatin structure. Slides were randomly batched and examined by the same investigator (CBA). Unlike absolute number of neutrophils in blood samples, absolute number of neutrophils in BALF is deceptive, because the technique yields a crude estimate, and many cells can be lysed in animals with severe inflammation. Because lysed cells would yield a low absolute cell count even in the face of severe inflammation, it was elected to use percentages for differential neutrophil counts. To numerically describe neutrophil percentages and degeneration in BALF samples, a score for BALF obtained from each calf was calculated by multiplying the percentage of neutrophils in the BALF by their grade for degeneration. An increase in neutrophil scores indicated an increase in neutrophil percentages, an increase in degeneration of neutrophils, or both, in BALF.

Bradford analysis of BALF—Bronchoalveolar lavage fluid from each neonatal calf was analyzed for protein content, using the Bradford method. Briefly, we prepared 8 dilutions of bovine serum albumin,[e] each of which was mixed with a constant amount of Bradford reagent dye[f] (2 ml of a 20% concentration) to provide solutions with final concentrations ranging from 0.1 to 1.0 mg/ml in a volume of 2.04 ml. These solutions were analyzed to create a standard curve, using a spectrophotometer[g] set at 595 nm. For determination of protein concentration, 40 µl of each BALF sample was added to 2 ml of 20% Bradford reagent dye. This mixture was analyzed, and the resultant concentration compared with values for the standard curve.

Detection of AAP in BALF—Concentration of AAP was determined in BALF obtained from each neonatal calf and adult cow by use of an ELISA, as described elsewhere.[32,38] Briefly, microtiter plate wells[h] were incubated overnight at 26 C with 100 µl (1 mg/ml) of ELISA-negative surfactant (negative-control sample) and ELISA-negative surfactant containing dilutions of synthesized AAP (positive-control sample) as well as 100 µl of BALF from each animal. Potential open reactive sites in wells were filled by incubating with blocking buffer (2 incubations for 30 minutes at 26 C). Wells were incubated with mouse anti-AAP antibody, 1G9-1C2, at a concentration of 1.0 µg/µl for 1 hour at 26 C. Wells then were washed twice with blocking buffer and incubated for 1 hour at 26 C with secondary antibody (goat anti-mouse IgG conjugated to peroxidase[i]), washed 3 times with blocking buffer, and incubated with developing reagent[j] for 5 minutes. Plates were read, using a spectrophotometer[a] set at 450 nm.

Antimicrobial activity of BALF supernatant—Antimicrobial activity for M haemolytica serotype A1 was determined for BALF obtained from each animal, using methods described elsewhere.[31,38,39] Briefly, M haemolytica serotype A1 was grown in tryptose broth at 37 C for 3 hours, pelleted by centrifugation (5,900 X g for 15 minutes at 4 C), and resuspended in saline solution. The suspension was adjusted, using a spectrophotometer[a] set at 600 nm, to 78% transmittance; this suspension contained 1×10^8 CFU/ml and was diluted with saline solution to achieve a concentration of 10^3 CFU/ml. A dilution susceptibility test was used to obtain the percentage of killed bacteria in BALF, compared with that in the saline control solution. Percentage of killed bacteria was determined by the following equation: (1 – [CFU in BALF/CFU in saline control mixture]) X 100.

Statistical analysis—An ANOVA[k] was used initially to determine significant differences among the 5 neonatal groups for band neutrophils in blood samples, BALF neutrophil scores, and BALF protein concentrations. When overall differences were detected among groups, pairwise comparisons of groups were performed. An ANOVA[k] also was used initially to determine significant differences among the 5 neonatal groups for BALF AAP concentrations and BALF antimicrobial activities. Because overall differences were not detected for those variables, t-tests were used for comparisons of the means for all neonatal calves with those for adult cattle. A value of $P < 0.05$ was considered significant.

Results

Neonatal calves—In all neonatal calves, WBC, differential counts, and fibrinogen concentrations differed between samples collected before and after inoculation. Changes were particularly evident in values for calves from the M haemolytica-inoculated groups and included decreased total leukocyte counts (including neutrophils and lymphocytes; data not shown). The number of band neutrophils was significantly higher in calves 6 hours after inoculation with M haemolytica, compared with values for calves from both control groups (2 and 6 hours after inoculation with saline solution) and for calves 2 hours after inoculation with M haemolytica (Table 1). The number of band neutrophils in TBC1269-treated calves 6 hours after inoculation with M haemolytica was similar to the values for calves from both control groups as well as for TBC1269-untreated calves 6 hours after inoculation with M haemolytica, and values were significantly ($P = 0.03$) higher, compared with values for calves 2 hours after inoculation with M haemolytica. We did not detect other notable alterations in blood values between TBC1269-treated and -untreated calves.

Table 1—Mean ± SE number of band neutrophils in blood samples obtained from neonatal calves before or after inoculation with *Mannheimia haemolytica*

Treatment	2 hours after inoculation ($\times 10^3$ cells/μl)			6 hours after inoculation ($\times 10^3$ cells/μl)		
	Before	After	Change	Before	After	Change
Saline solution	0.09 ±0.09	0.14 ± 0.11	0.04 ± 0.17*	0.03 ± 0.03	0.11 ± 0.11	0.07 ± 0.13*
Mannheimia haemolytica	0.06 ± 0.02	0.02 ± 0.02	−0.03 ± 0.04*†	0.33 ± 0.33	1.14 ± 0.51	0.81 ± 0.43
Mannheimia haemolytica + TBC1269	ND	ND	ND	0.22 ± 0.15	0.75 ± 0.22	0.53 ± 0.20

*Value is significantly ($P < 0.05$) less than the value for 6 hours after inoculation with *M haemolytica*. †Value is significantly ($P < 0.05$) less than the value for 6 hours after inoculation of calves with *M haemolytica* that were treated with TBC1269. Saline = Inoculated with 5 ml of pyrogen-free saline (0.14*M* NaCl) solution; n = 3 at 2 hours and 3 at 6 hours. *Mannheimia haemolytica* = Inoculated with 5 ml of solution that contained 1 × 10⁸ colony forming units (CFU) of *M haemolytica*/ml; n = 4 at 2 hours and 4 at 6 hours. *Mannheimia haemolytica* + TBC1269 = Treated with a selectin inhibitor (TBC1269; 25 mg/kg of body weight, IV) 30 minutes before and 2 hours after being inoculated with 5 ml of solution that contained 1 × 10⁸ CFU of *M haemolytica*/ml; n = 4. ND = Not determined.

Table 2 —Percentage of neutrophils, grade for neutrophil degeneration, and neutrophil scores for bronchoalveolar lavage fluid (BALF) obtained from neonatal calves

Treatment	Percentage of neutrophils	Grade for neutrophil degeneration*	Score†
Saline solution			
2 hours after inoculation	20 to 81	0 to 3	0 to 0.03
6 hours after inoculation	80 to 95	0 to 1	0 to 0.2
Mannheimia haemolytica			
2 hours after inoculation	65 to 90	0.5 to 4	0.33 to 1.1
6 hours after inoculation	60 to 100	1.5 to 4	1.35 to 4
Mannheimia haemolytica + TBC1269			
6 hours after inoculation	60 to 99	0 to 4	1.3 to 2.7

Values represent ranges for all calves in each group. *Scale of 0 (nondegenerate) to 4 (severely degenerate). †Score was calculated by multiplying percentage of neutrophils by grade for neutrophil degeneration. *See* Table 1 for remainder of key.
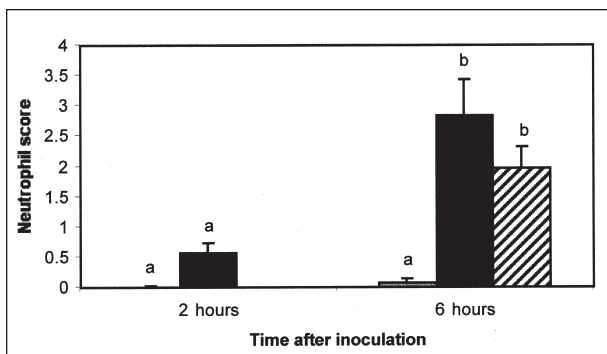

Figure 1—Mean ± SE neutrophil scores in bronchoalveolar lavage fluid (BALF) obtained from neonatal calves. Calves were treated as follows: inoculated with 5 ml of pyrogen-free saline (0.14*M* NaCl) solution (gray bar), inoculated with 5 ml of solution that contained 1 × 10⁸ colony forming units (CFU) of *Mannheimia haemolytica*/ml (black bar), or treated with a selectin inhibitor (TBC1269; 25 mg/kg of body weight, IV) 30 minutes before and 2 hours after being inoculated with 5 ml of solution that contained 1 × 10⁸ CFU of *M haemolytica*/ml (striped bar). a,b = Values with different letters differ significantly ($P < 0.05$).
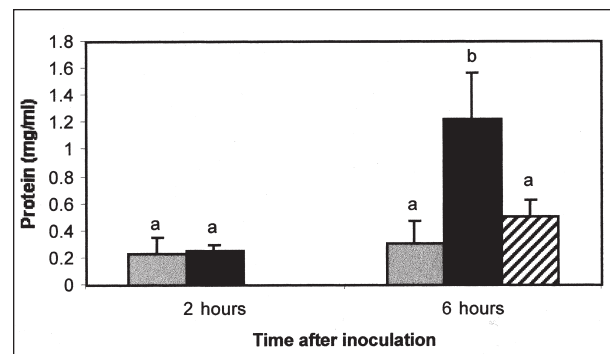

Figure 2—Mean ± SE protein concentrations in BALF obtained from neonatal calves. *See* Figure 1 for key.

BALF neutrophil scores—Increased neutrophil scores indicated an increase in BALF neutrophil percentages, an increase in degeneration of neutrophils, or both. For all calves, the factor that had the most effect on neutrophil score was the degree of degeneration, rather than neutrophil percentage (**Table 2**). Neutrophil percentage was similar among calves from all groups. Bronchoalveolar lavage fluid from calves from both control groups as well as calves 2 hours after inoculation with *M haemolytica* had significantly lower neutrophil scores, compared with scores for TBC1269-treated and -untreated calves 6 hours after inoculation with *M haemolytica* (**Fig 1**). Neutrophil scores for calves 2 hours after inoculation with *M haemolytica* were higher, but not significantly, than neutrophil scores for both control groups. Neutrophil scores for calves 6 hours after inoculation with *M haemolytica* that were treated with TBC1269 were lower, but not significantly ($P = 0.11$), than values for calves 6 hours after inoculation with *M haemolytica* that were not treated with TBC1269.

BALF protein concentration—Protein concentration in BALF from calves of both control groups and calves 2 hours after inoculation with *M haemolytica* were significantly lower, compared with concentrations for TBC1269-untreated calves 6 hours after inoc-

Table 3—Antimicrobial anionic protein (AAP) concentrations and minimum inhibitory concentrations (MIC) in BALF obtained from neonatal calves and adult cows

	AAP (mM)		MIC (%)	
Treatment	2 hours after inoculation	6 hours after inoculation	2 hours after inoculation	6 hours after inoculation
Calves (n = 18)				
Saline	0.32 ± 0.17	0.37 ± 0.26	9.7 ± 9.7	0 ± 0
Mannheimia haemolytica	0.36 ± 0.05	0.35 ± 0.1	1.8 ± 1.8	0 ± 0
Mannheimia haemolytica +				
TBC1269	ND	0.20 ± 0.06	ND	0 ± 0
All calves	0.32 ± 0.05[a]		2.0 ± 1.6[a]	
Cows (n = 9)	0.11 ± 0.05[b]		62.7 ± 5.8[b]	

Values reported are mean ± SE. [a,b] = For each variable, values differ significantly ($P < 0.05$) between BALF obtained from adult and neonatal cattle. *See* Table 1 for remainder of key.

ulation with *M haemolytica* (**Fig 2**). In addition, protein concentrations for TBC1269-treated calves 6 hours after inoculation with *M haemolytica* were significantly lower than for TBC1269-untreated calves.

Concentration of AAP in BALF—Antimicrobial anionic peptide was detected in BALF from all neonatal calves (mean ± SE, 0.32 ± 0.05 mM) and adult cattle (0.11 ± 0.05 mM; **Table 3**). Values for neonates were significantly ($P = 0.02$) higher than for adults; however, we did not detect significant differences in AAP concentrations among neonatal groups.

Antimicrobial activity of BALF supernatant—With the exception of 2 calves (1 in each of 2 groups), BALF from neonatal calves lacked detectable antimicrobial activity, whereas BALF from adult cattle had substantial antimicrobial activity (**Table 3**). Percentage killing of *M haemolytica* achieved by use of BALF from adult cattle ranged from 32 to 84% (mean, 62.7 ± 5.8%). In contrast, percentage killing achieved by use of BALF from neonates ranged from 0 to 29% (mean, 2.0 ± 1.6%). The percentage killing achieved by use of BALF from adult cattle was significantly ($P = 0.01$) higher than that for BALF from neonates, even though neonatal BALF contained more AAP.

Discussion

Values changed for blood samples from all groups of neonatal calves after inoculation in a manner that would be expected in animals undergoing stress or tissue demand for WBC. These changes included decreased total leukocyte counts, including neutrophils and lymphocytes, and an increased number of band neutrophils. Many of these changes were not significantly different until 6 hours after inoculation with *M haemolytica*, which was attributed to the time lag between initial pulmonary inoculation with *M haemolytica* and the leukocyte response in the circulation. Band neutrophil counts in blood samples of calves obtained 2 hours after inoculation with *M haemolytica* were decreased, compared with counts before inoculation. This decrease did not differ significantly from changes found in either group of control calves (2 or 6 hours after inoculation with saline solution) but did differ significantly from changes in the TBC1269-treated group. This reflects the lack of a leukocyte response in the circulation by 2 hours after inoculation with *M haemolytica* as well as variability in

leukocyte counts of neonates. We did not detect any other significant differences in blood leukocyte counts between the TBC1269-treated group and any other group; therefore, differences in BALF neutrophil counts between calves 6 hours after inoculation with *M haemolytica* that were treated or not treated with TBC1269 probably were attributable to alterations in neutrophil activity in the pulmonary vasculature, rather than the drug's ability to inhibit leukocyte production or to sequestration of leukocytes.

A high neutrophil score reflects a high neutrophil percentage in the BALF, a high degree of neutrophilic cellular degeneration and lysis, or both. All calves had similar total percentages of neutrophils as a result of the inflammatory response elicited by deposition of a fluid substance (saline solution or *M haemolytica*) within a bronchus. Absolute neutrophil counts in BALF could not be accurately assessed, but, one of the investigators (ZAR) determined by use of morphometric assessment that neutrophilic infiltration into pulmonary spaces was significantly reduced in the lungs of calves 6 hours after inoculation with *M haemolytica* that were treated with TBC1269, compared with results for samples obtained from calves that were not treated with TBC1269. Similar decreases in neutrophil infiltration have been documented with the use of TBC1269 in BALF obtained from sheep as well as rat liver.[23,25]

Despite the reduction in neutrophil infiltration, differences in neutrophil scores among groups were attributable largely to differences in degree of neutrophil degeneration. Bronchoalveolar lavage fluid that contains an increased number of degenerate neutrophils is indicative of a more severe pulmonary inflammatory response. Furthermore, this indicates a greater number of lysed neutrophils that release neutrophilic enzymes, resulting in damage to pulmonary tissue. In support of another study that documented substantial inflammation in the lungs by 4 hours after inoculation with *M haemolytica*,[16] neutrophil scores in BALF were significantly ($P = 0.01$) higher in samples obtained from our calves 6 hours after inoculation with *M haemolytica* that were not treated with TBC1269, compared with samples obtained 2 hours after inoculation. Neutrophil scores for calves 2 hours after inoculation with *M haemolytica* were not significantly different than values for either group of control calves, again supporting the lack of substantial inflammation

by 2 hours after inoculation with *M haemolytica*. Lower scores were seen in BALF obtained from calves 6 hours after inoculation with *M haemolytica* that were treated with TBC1269, but they were not significantly (P = 0.11) different, compared with scores of calves that were not treated with TBC1269. This was largely attributable to decreased degeneration of neutrophils in calves given TBC1269.

Neutrophil migration into the lungs during *M haemolytica*-induced pneumonia may be beneficial or deleterious. During pneumonia attributable to *M haemolytica*, neutrophils are needed to reduce bacterial viability, yet release of cellular lysosomal enzymes, free radicals, and other factors contributes to tissue damage.[8-10] Protein leakage from vessels as a result of pulmonary parenchymal and vascular compromise (damage to the alveolar-capillary barrier) is a measure of tissue damage.[40,41] In the previously mentioned study,[16] a significant number of neutrophils migrated into the lungs by 4 hours after inoculation with *M haemolytica*, coincident with a significant increase in protein concentrations in the BALF. In support of that study, BALF protein concentrations reported here were significantly (P = 0.003) higher 6 hours after inoculation with *M haemolytica* from TBC1269-untreated calves, compared with values 2 hours after inoculation. Protein concentrations from calves 2 hours after inoculation with *M haemolytica* did not differ significantly from values for either group of control calves, which further supports a lack of substantial inflammation 2 hours after inoculation with *M haemolytica*. Bronchoalveolar lavage fluid from calves 6 hours after inoculation with *M haemolytica* that were treated with TBC1269 had significantly (P = 0.02) lower concentrations of protein, compared with concentrations in BALF obtained from calves not treated with TBC1269. This result supports results of studies in which there was a decrease in tissue damage and vascular leakage of protein following neutrophil depletion.[13-16]

These findings for protein concentration in BALF reflect changes seen in neutrophil scores. Neutrophils that are more degenerate are indicative of a more severe inflammatory process, and this results in a release of additional neutrophilic enzymes, causing additional pulmonary tissue damage with breakdown of the alveolar capillary barrier and protein leakage. Use of TBC1269 caused significantly (P = 0.02) lower protein concentrations in BALF, compared with concentrations in BALF from calves not treated with TBC1269, indicating less pulmonary tissue injury in TBC1269-treated calves. This indicates a lower degree of inflammatory response in these calves that may be attributable to several mechanisms. First, TBC1269 inhibits selectin-mediated adherence between neutrophils and endothelium, and this may reduce the number of neutrophils that pass through pulmonary vessels and enter pulmonary spaces. Because we could not assess BALF absolute cell counts, decreased infiltration of neutrophils into pulmonary spaces could not be assessed, but it was seen during morphometric assessment of these tissues (ZAR) and has been reported with the use of TBC12689 in BALF obtained from sheep[25] as well as rat liver.[23] Second, TBC1269 may alter or reduce neutrophil activation

through inhibition of selectin-ligand binding. During neutrophil infiltration into the lungs, selectin-ligand binding activates neutrophils to express β_2-integrins that bind intracellular adhesion molecules (ICAM), mediating vascular extravasation and tissue migration.[21] Lack of activation may prevent optimal β_2-integrin expression, thus further interfering with neutrophilic infiltration into pulmonary spaces. Third, neutrophil activation is followed by metalloproteinase cleavage of the L-selectin molecule at the point where the external domain joins the transmembrane domain.[42] The internal cytoplasmic tail of L-selectin interacts with cytoskeletal elements, including α-actinin.[42] It is possible that decreased selectin-ligand binding maintains the integrity of the selectin molecule and its connection to cytoskeletal elements. This may explain the lower amount of neutrophilic degeneration in treated calves. Fourth, *M haemolytica* leukotoxin may promote neutrophil apoptosis by binding to β_2-integrin.[43] Prevention of selectin-ligand-mediated activation of β_2-integrin expression may prevent leukotoxin binding, providing an indirect means for prevention of neutrophil apoptosis. Last, the kinetics of TBC1269 have not been fully explored in cattle, which is attributable to the fact that it is an experimental compound. However, in 1 study, TBC1269 administered once (3 mg/kg, IV) caused reduced neutrophil infiltration in sheep.[25] Although the dosage used in the study reported here was considered extremely high, and the dosing time was considered repetitious, there is the possibility that the compound is not effective by 6 hours after administration. In that case, the low number of mildly degenerate cells in BALF from TBC1269-treated calves may have migrated from the vasculature into the airways only a short time before BALF was harvested and would, at a later time point, resemble those in the untreated calves. Thus, barring erroneous interpretation due to inadequate dosing of TBC1269, selectin inhibition of nuetrophils appears to prevent a portion of the nuetrophil-mediated damage to the pulmonary tissues, thereby maintaining the protective pulmonary epithelial barrier and preserving epithelial defense mechanisms.

Experimental depletion of neutrophils during *M haemolytica*-induced pneumonia results in a reduction in tissue damage and vascular leakage of protein.[13-16] However, *M haemolytica* can induce pulmonary tissue damage independent of neutrophils. *Mannheimia haemolytica* endotoxin (lipopolysaccharide) can have cytotoxic effects on endothelial cells and can produce pulmonary lesions.[5,44,45] Also, *M haemolytica* has additional products, such as leukotoxin and capsular polysaccharide, capable of producing tissue damage.[5,37,45,46] Thus, complete neutrophil depletion is not a viable therapeutic alternative for calves with pneumonia attributable to *M haemolytica* in terms of practicality or in effectiveness at clearing infection and preventing related tissue damage. Selectin inhibition may provide a means to decrease but not completely block neutrophil infiltration from the vasculature into the pulmonary spaces in response to infection with *M haemolytica*. Some degree of neutrophil infiltration into pulmonary spaces through thin alveolar walls probably is retained following TBC1269 administration.

Large amounts of AAP are detectable in BALF from humans and sheep[31,32]; however, to our knowledge, this is the first report of the detection of AAP in BALF from neonatal calves. Antimicrobial anionic peptide was evident at similar concentrations in the BALF from calves of all groups. Significant differences were not detected in concentrations from control calves, calves that had been inoculated with *M haemolytica*, or calves that had been inoculated with *M haemolytica* and treated with TBC1269. Mean AAP concentration in BALF obtained from all neonatal calves was approximately 3-fold higher than the mean AAP concentration in BALF obtained from adult cattle.

Even though mean AAP concentration in BALF from adult cattle was approximately a third of the mean concentration in BALF from neonates, BALF from neonates containing this peptide had significantly less antimicrobial activity against *M haemolytica*, compared with the activity of BALF from adults. Antimicrobial activity was discernible in BALF from only 2 calves. Values for these 2 calves may have represented sample error, or there may have been antimicrobial activity attributable to AAP or an alternative antimicrobial substance in these young animals. It is believed that AAP is a propeptide that is cleaved from a larger peptide.[31] Thus, it is possible that the larger peptide is not being effectively produced or metabolized in neonatal calves, and, therefore, neonatal calves lack antimicrobial activity. Also, antimicrobial peptides work in concert with other compounds and substances in vivo to kill microbes effectively.[47] It is possible that unknown cofactors or copeptides necessary for full antimicrobial activity of AAP may not exist or may not be effective in neonatal animals.

Although AAP is microcidal alone or in concert with surfactant,[31] surfactant proteins and other molecules in BALF also are microcidal[48] and may result in the antimicrobial activity detected in BALF from adult and neonatal cattle. Thus, although it is possible that the differences in antimicrobial activity between adults and neonates seen in this study were related to the function of AAP or its regulators, the effectiveness of AAP alone in bovine BALF cannot be assessed in our study. Additional investigations are needed to directly assess antimicrobial activity of AAP in bovine BALF.

In neonates, *M haemolytica*-induced pneumonia does not appear to reduce the concentration of AAP in BALF. Perhaps AAP simply was not being consumed in fighting the infection, because it was not antimicrobially active at that time. This also could explain the lower basal concentrations of AAP in BALF of adult cattle, compared with values for BALF of neonates. Antimicrobial peptides function in response to microbial infection, but they also prevent initial colonization.[49] Antimicrobial anionic peptide may be continually consumed in adult animals in a prophylactic manner, whereas BALF concentrations in neonates remain high, because the peptide lacks function. This lack of AAP activity may help explain the sensitivity of neonates to pulmonary colonization with infectious organisms such as *M haemolytica*.

Selectin inhibition resulted in decreased neutrophil-mediated damage to pulmonary tissues, as evidenced by decreased vascular leakage of protein into BALF in TBC1269-treated calves 6 hours after inoculation with *M haemolytica*. Thus, TBC1269 may be an effective therapeutic tool for pulmonary infections such as *M haemolytica*-induced pneumonia, because neutrophils retain their ability to infiltrate pulmonary spaces, although fewer infiltrate. Neutrophils are necessary to combat infection, so a drug that only partially blocks neutrophilic infiltration into pulmonary spaces may be effective in allowing some neutrophils into these areas while concurrently limiting their number to prevent excessive neutrophil-mediated tissue damage. Additional studies could include evaluation of the use of TBC1269 in conjunction with an antibiotic in the treatment of calves with acute pneumonia attributable to *M haemolytica* to determine whether a beneficial synergistic effect exists between the compound and an antibiotic. In addition, TBC1269 is effective in preventing neutrophil-mediated reperfusion injury in rat liver[23] and in reducing the initial cellular response to pulmonary antigen in a sheep model of allergic bronchoconstriction.[25] Thus, TBC1269 may be effective in the treatment of multiple species of animals with several types of inflammatory disorders.

Neonatal calves appear to lack a mature effective antimicrobial peptide response, as evidenced by the decreased bactericidal activity of BALF obtained from neonates, compared with activity of BALF obtained from adult cattle, despite a concentration of AAP in neonatal BALF at 3 times the concentration found in BALF of adult cattle. We believe that this may be a reason for the susceptibly of neonatal calves to pulmonary infections. The potential role of AAP in the prevention and treatment of naturally acquired disease requires additional investigation.

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