Effects of the synthetic selectin inhibitor TBC1269 on tissue damage during acute Mannheimia haemolytica-induced pneumonia in neonatal calves

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Objective—To determine effects of the selectin inhibitor TBC1269 on neutrophil-mediated pulmonary damage during acute Mannheimia haemolytica-induced pneumonia in newborn calves.

Animals—Eighteen 1- to 3-day-old colostrum-deprived calves.

Procedure—Mannheimia haemolytica or saline (0.9% NaCl) solution was inoculated in both cranial lung lobes of 12 and 6 calves, respectively. Calves were euthanatized 2 (saline, n = 3; M haemolytica, n = 4) or 6 hours (saline, n = 3; M haemolytica, n = 8) after inoculation. Four M haemolytica-inoculated calves euthanatized at 6 hours also received TBC1269 (25 mg/kg, IV) 30 minutes before and 2 hours after inoculation. Conjugated diene (CD) concentrations, inducible nitric oxide synthase (iNOS) expression, and apoptotic cell counts were determined in lung specimens collected during necropsy.

Results—Conjugated diene concentrations were significantly increased in all M haemolytica-inoculated groups, compared with saline-inoculated groups. Calves treated with TBC1269 had decreased concentrations of CD, compared with untreated calves, although the difference was not significant. Number of apoptotic neutrophils and macrophages increased significantly in TBC1269-treated calves, compared with untreated calves. Inducible nitric oxide synthase was expressed by epithelial cells and leukocytes. However, iNOS was less abundant in airway epithelial cells associated with inflammatory exudates. Degree of iNOS expression was similar between TBC1269-treated and untreated calves.

Conclusions—Mannheimia haemolytica infection in neonatal calves resulted in pulmonary tissue damage and decreased epithelial cell iNOS expression. The selectin inhibitor TCB1269 altered, but did not completely inhibit, neutrophil-mediated pulmonary damage. (Am J Vet Res 2001;62:17–22)

Infiltration of neutrophils into sites of infection provides an important defense mechanism against bacterial pathogens.1 Infiltration into tissues is a multistep process involving the coordinated function of multiple families of adhesion molecules, cytokines, and chemoattractants.2 Initial adherence of leukocytes to endothelium is a loose interaction between cell-surface selectins (E-, P- and L-selectin) and ligands on endothelial cells.

This loose interaction is followed by firm adherence, which is mediated by upregulation of β2-integrins (eg, CD11b/CD18) on leukocytes and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.2,3 Selectins bind to various ligands on vascular endothelial cells, including CD34, P-selectin glycoprotein ligand-1 (PSGL-1), glycoprotein cell adhesion molecule-1 (GlyCAM-1), and sialyl Lewis (sLe)2. Each of the 3 selectins binds to sLe2.4 After cytokine-mediated activation of leukocytes, L-selectin is shed from neutrophils, and β2-integrins are activated to mediate tight adhesion of neutrophils to ICAM-1. This, in turn, is followed by transendothelial migration of neutrophils.

Accumulation of neutrophils in tissues has a central role in many inflammatory conditions, including that which develops during acute lung and reperfusion injuries.1 Acute pulmonary pasteurellosis is a common respiratory disease of cattle caused by infection with Mannheimia haemolytica (formerly known as Pasteurella haemolytica) and characterized by a dense and often excessive infiltration of neutrophils into the lungs.5 These infiltrates are associated with extensive parenchymal necrosis. Tissue damage is attributable, in part, to bacterial products such as leukotoxin and lipopolysaccharides (LPS).6,7 Wang et al7 demonstrated that M haemolytica leukotoxin binds to β2-integrins on leukocytes and induces apoptosis of leukocytes. In addition, oxygen-derived free radicals, elastases, cytokines, and acid hydrolases produced by actively degranulating neutrophils are cytotoxic to vascular endothelium and contribute to pulmonary damage and leakage of proteins.8,9 Nitrite concentrations in bronchoalveolar lavage fluid from calves infected with M haemolytica are significantly increased, compared with noninfected calves, suggesting a role of nitric oxide (NO) in the pathogenesis of pneumonic pasteurellosis.9 However, expression and regulation of inducible nitric oxide synthase (iNOS) in bovine lung epithelium during acute pulmonary pasteurellosis has not been assessed. Depletion of neutrophils in calves experimentally infected with M haemolytica significantly reduces the severity of pulmonary lesions.10,11 Therefore, understanding the early events of neutrophil-mediated damage to respiratory tissue during M haemolytica infection may enhance the ability to control the deleterious and promote the beneficial effects of acute inflammation.

Inhibition of selectin binding to ligands such as sLe2 may have therapeutic effects by inhibiting neutrophil infiltration and subsequent tissue damage. Monoclonal antibodies, synthetic sulfatides, and sLe2 analogs have been used to inhibit selectin binding in various animals with experimentally induced lung injury.12,13 However, to our knowledge, selectin inhibition in experimentally
induced pulmonary pasteurellosis in cattle has not been studied. A small molecule synthetic selectin inhibitor, TBC1269, has been shown to inhibit sLeα-dependent binding and binding of leukocytes to P-, E-, and L-selectin in vitro. This compound has also been tested in sheep with experimentally induced allergic pulmonary disease. Treatment with TBC1269 resulted in significant protection against allergic airway responses. We hypothesized that inhibition of selectin by use of TBC1269 in calves with pulmonary pasteurellosis would attenuate or inhibit neutrophil-mediated damage to pulmonary tissue. The purpose of the study reported here was to determine the effects of TBC1269 on neutrophil-mediated pulmonary damage in acute M haemolytica pneumonia in newborn calves.

Materials and Methods

Experimental design—Protocols for these experiments were approved by the Iowa State University Animal Care Committee. Eighteen 1- to 3-day-old colostrum-deprived Holstein-Friesian calves were randomly assigned to 1 of 5 groups. Six calves were inoculated with saline (0.9% NaCl) solution via a fiber-optic bronchoscope placed in right and left cranial lung lobes and euthanatized either 2 hours (n = 3) or 6 hours (n = 3) later. Twelve calves were inoculated with 5 ml of M haemolytica (1 × 10^6 colony forming units [CFU/ml]) in a similar fashion. This number of bacteria results in development of consistent pulmonary pasteurellosis lesions. One group of M haemolytica-inoculated calves was euthanatized 2 hours after inoculation (n = 4). One group was euthanatized 6 hours after inoculation (n = 4), and 1 group was treated with TBC1269 (25 mg/kg of body weight, IV) 30 minutes before and 2 hours after inoculation (n = 4). This latter group was euthanatized 6 hours after inoculation. Pulmonary tissue specimens were collected during necropsy from all calves for evaluation of tissue damage.

Inoculations—Mannheimia haemolytica serovar 1 was grown overnight on blood agar, transferred to trypticase soy broth and incubated 1.3 to 2.5 ml in 0.14 M NaCl solution for a fiber-optic bronchoscope placed in right and left cranial lung lobes and euthanatized either 2 hours (n = 3) or 6 hours (n = 3) later. Twelve calves were inoculated with 5 ml of M haemolytica (1 × 10^6 CFU/ml) in a similar fashion. This number of bacteria results in development of consistent pulmonary pasteurellosis lesions. One group of M haemolytica-inoculated calves was euthanatized 2 hours after inoculation (n = 4). One group was euthanatized 6 hours after inoculation (n = 4), and 1 group was treated with TBC1269 (25 mg/kg of body weight, IV) 30 minutes before and 2 hours after inoculation (n = 4). This latter group was euthanatized 6 hours after inoculation. Pulmonary tissue specimens were collected during necropsy from all calves for evaluation of tissue damage.

Preparation of TBC1269—TBC1269 (1,6-Bis[3-(3-carboxymethylphenyl)-4-(2-g-D-mannopyranosylxy) phenyl]hexane), a synthetic small molecule nonoligosaccharide selectin inhibitor was provided in a powder form. This inhibitor inhibits binding of E-, P-, and L-selectins to sLeα, with inhibitory concentration 50 (IC50) values of 105 μM, 17 μM, and 87 μM, respectively. Results of safety and efficacy studies indicate that TBC1269 does not have cytotoxic effects. TBC1269 has been used in humans with allergic asthma. Treatment resulted in a reduction in number of eosinophils in sputum and improvement of the late bronchoconstriction reaction.

For administration of TBC1269 to calves, the required dose was prepared in sterile pyrogen-free saline solution. Approximately 9 ml of saline solution (pH 7.4) was added to 1 g of TBC1269 powder. The dose used (25 mg/kg, IV) was chosen on the basis of results of human clinical trials performed by the manufacturer. Timing of administration of TBC1269 (30 minutes before and 2 hours after inoculation with M haemolytica) was chosen to obtain maximal inhibition of selectins.

Specimen collection—Calves were euthanatized by IV injection of an overdose of sodium pentobarbital immediately prior to tissue collection. Specimens of lung tissues were collected from the central area of the inoculation site, and included an area of tissue no less than 2 × 2 cm. Tissue specimens were divided into several smaller sections for determination of conjugated diene (CD) concentrations and detection of apoptotic cells and iNOS-producing cells. For detection of apoptotic cells and iNOS-producing cells, specimens were fixed in neutral-buffered 10% formalin, and for determination of CD concentrations, specimens were placed in 1.8-ml vials and frozen in liquid nitrogen.

Determination of conjugated diene concentration—As a measure of cell membrane damage in response to inoculation with M haemolytica and after treatment with TBC1269 treatment, CD concentrations were determined by use of a commercially available colorimetric assay according to the manufacturer’s instructions. Briefly, approximately 2 to 3 g of tissue was washed in ice-cold saline solution, blotted, and minced in ice-cold 20 mM Tris-HCl buffer, pH 7.4. Minced tissue was diluted with ice-cold Tris-HCl buffer to approximately 20 to 30% (2 to 3 g/10 ml), homogenized, and centrifuged at 5,000 × g at 4°C for 25 minutes. Supernatant (approx 200 μl) was collected and assayed according to instructions. Optical density at 586 nm was determined spectrophotometrically and concentrations of CD calculated by comparison with a standard curve. Samples were normalized to protein concentration by use of a modified Bradford protein assay. Protein concentrations in the supernatant of unknown samples were determined spectrophotometrically at a wavelength of 595 nm and by comparison to a bovine serum albumin standard.

In situ detection of apoptotic cells—We used the terminal deoxynucleotidyl transferase-mediated dUTP nick

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Time (h)*</th>
<th>CD (μM/mg of protein)</th>
<th>Apoptotic cells (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (3)</td>
<td>2</td>
<td>7.3 ± 0.78</td>
<td>4.4 ± 0.25</td>
</tr>
<tr>
<td>M haemolytica (4)</td>
<td>2</td>
<td>13.7 ± 1.68</td>
<td>8.1 ± 0.77</td>
</tr>
<tr>
<td>Saline (3)</td>
<td>6</td>
<td>11.8 ± 0.35</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>M haemolytica (4)</td>
<td>6</td>
<td>20.0 ± 2.3</td>
<td>8.2 ± 2.1</td>
</tr>
<tr>
<td>TBC1269-treated</td>
<td>6</td>
<td>16.8 ± 1.7</td>
<td>12.2 ± 1.3⁸</td>
</tr>
</tbody>
</table>

Data reported as mean ± SEM.

*Time after inoculation.

Significantly (P < 0.05) greater than value for saline group, 2 hours after inoculation. Significantly (P < 0.05) greater than value for saline group, 6 hours after inoculation. Significantly (P < 0.05) greater than value for M haemolytica group, 6 hours after inoculation. Significantly (P < 0.05) greater than value for saline group, 6 hours after inoculation.
end-labeling (TUNEL) technique to detect apoptotic cells and examine the effects of TBC1269 on apoptosis. A TUNEL assay was performed, using a commercially available kit according to the manufacturer’s instructions with slight modifications. Five-micron-thick sections were cut from tissue specimens and placed on silane-coated slides. These sections were dewaxed at 58°C for 30 minutes, deparaffinized in 2 changes (5 minutes each) of xylene, and hydrated through graded alcohol baths to ultrapure water. Sections were washed with Tris-HCl buffer, treated with proteinase K (10 µg/ml) for 30 minutes at 37°C, and rinsed 2 times in Tris-HCl buffer. Sections were incubated with 0.3% hydrogen peroxide in a methanol blocking solution for 30 minutes to inactivate endogenous peroxidase, followed by 2 washes in Tris-HCl buffer. Sections were then incubated with terminal deoxynucleotidyl transferase and a mixture of nucleotides for 1 hour at 37°C. Negative control sections were incubated with the nucleotide mixture only. After washing, sections were incubated with peroxidase for 1 hour at room temperature (25°C), washed 2 times, incubated with diaminobenzidine for 5 minutes, and counterstained with hematoxylin. Apoptotic (ie, labeled) cells in 20 HPF (400X; equivalent to 3.694 mm²) were counted and expressed as cells/mm².

Immunohistochemistry—Detection of 3-nitrotyrosine (nitrotyrosine) has been used as an indicator of in vivo iNOS expression. Expression and distribution of iNOS during acute M haemolytica pneumonia and after treatment with TBC1269 treatment were assessed by use of immunohistochemistry. The immunohistochemistry procedure was similar to a described technique with minor modifications. Sections of lung were prepared as for detection of apoptotic cells. After hydration through graded alcohol baths to ultrapure water, sections were treated with 3% hydrogen peroxide-methanol for 3 hours to inactivate endogenous peroxidase. Sections were washed in 1X buffer, incubated with 300 µl of human IgG (8 mg/ml) for 30 minutes at room temperature, and washed twice in 1X buffer. Sections were then incubated with 300 µl of rabbit anti-nitrotutrosine polyclonal antibody (diluted 1:100 with reagent diluent) at 37°C for 1.5 hours in a humidified chamber, washed in 1X buffer, and incubated with 300 µl of horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:200 with reagent diluent) for 45 minutes at 37°C. After 3 washes in 1X buffer, sections were incubated with 3-amino-9-ethylcarbazole for 5 to 10 minutes at room temperature and counterstained with hematoxylin. Permanent aqueous mounting medium was applied to each section, and sections were placed in an oven at 38°C for 1 hour. Postmounting medium was placed on each slide, and slides were coverslipped. Control reactions performed to confirm the specificity of the primary antibody included sections incubated with the omission of primary antibody; sections incubated with normal rabbit serum instead of the primary antibody; and sections incubated with primary antibody that was preabsorbed with 1 mmol of nitrotyrosine/L. The intensity of iNOS staining was measured, using a staining score from 0 to 3 (0 = no staining; 1 = weak staining in < 30% of cells; 2 = moderate staining in 30 to 60% of the cells; 3 = intense staining in > 60% of the cells).

Statistical analyses—All numerical data were reported as mean ± SEM. Differences among treatments (saline-inoculated, M haemolytica-inoculated [untreated], or M haemolytica-inoculated and TBC1269-treated [TBC1269-treated]) at 2 or 6 hours after inoculation were analyzed by use of ANOVA. Differences were considered significant when P ≤ 0.05.

Results

Effects of M haemolytica inoculation—After inoculation with M haemolytica, calves were lethargic, had moderate difficulty breathing, and became recumbent.
bronchopneumonia was detected in tissue specimens from all *M. haemolytica*-inoculated calves. Moderate to severe multifocal infiltrates of degenerate neutrophils (ie, streaming leukocytes) admixed with large amounts of fibrin, edema fluid, and some necrotic cell debris were evident. These infiltrates were detected in alveoli, bronchi, bronchioles, and interstitial spaces. Mild interstitial edema was the only lesion detected in lung specimens from saline-inoculated calves.

**Effect of TBC1269 on conjugated diene concentrations**—Conjugated diene concentrations in lung tissue were compared among groups. Concentrations in all groups of *M. haemolytica*-inoculated calves were significantly increased 2 and 6 hours after inoculation, compared with saline-inoculated calves. Conjugated diene concentration in the TBC1269-treated group was decreased, compared with concentration in the untreated group. However, this difference was not significant (Table 1).

**Effect of TBC1269 on number of apoptotic cells**—Apoptotic cell counts (neutrophils and macrophages) 6 hours after inoculation were greater in the TBC1269-treated group than in the other groups (Fig 1 and Table 1). In addition, apoptotic cell counts 2 and 6 hours after inoculation were greater in the untreated groups, compared with the saline-inoculated groups.

**Inducible nitric oxide synthase expression**—Cells expressing iNOS were evident in lung tissue from saline-inoculated calves 2 and 6 hours after inoculation. Staining was moderate (score 2) to strong (score 3) and was localized in the pulmonary bronchiolar and alveolar epithelial cells, bronchial cartilage, alveolar macrophages, and smooth muscle cells (Fig 2). Staining was abolished when lung sections were incubated with anti-nitrotyrosine antibody preabsorbed with an excess of nitrotyrosine or when no primary antibody was added. In sections from untreated and TBC1269-treated calves, areas adjacent to inflamed and unaffected regions had a similar (moderate to strong) staining pattern to that in sections from saline-inoculated calves. Widespread but weaker staining (score 1) was evident in consolidated areas of lung tissue. However, strong staining (score 3) was evident in individual multifocal infiltrates of leukocytes (neutrophils and macrophages) in these areas.

**Discussion**

The acute inflammatory reaction that develops during infection with *M. haemolytica* is an adaptive response that can reduce bacterial colonization; however, in severe, acute bacterial pneumonia of neonates, it is often excessive and results in tissue damage. In this study, we investigated the in vivo effects of TBC1269, a synthetic nonoligosaccharide selectin inhibitor, as a potential treatment for excessive acute inflammation in neonatal calves.

Oxygen-derived free radicals, generated mainly by phagocytic cells, have been proposed to cause tissue injury during endotoxemia. These metabolites may cause tissue damage by initiating lipid peroxidation that leads to alteration in cell membrane function and structure. Conjugated dienes, such as malondialdehyde and 4-hydroxy-2(E)-nonenal, are recognized markers of lipid peroxidation and tissue damage. In this study, all *M. haemolytica*-inoculated calves had significantly greater CD concentrations in lung tissue 2 and 6 hours after inoculation, compared with saline-inoculated calves. This result is in agreement with results of another study in which increased lipid peroxidation was detected after pulmonary administration.
of endotoxin. The extent of tissue damage (as measured by CD production) in TBC1269-treated calves was reduced, compared with untreated calves; however, the reduction in damage was not significant.

Several possibilities may explain the inability of TBC1269 to significantly decrease CD formation. We evaluated only the acute inflammatory phase (2 and 6 hours) of M haemolytica infection. More time may be required to completely block all selectin molecules with TBC1269 and reduce tissue damage. Attempts to evaluate calves more than 6 hours after inoculation were unsuccessful because of the death of calves after the 6-hour time point. Pulmonary pasteurellosis is difficult to reproduce experimentally, and we have found that a high bacterial concentration is needed to induce consistent lesions. Therefore, we were unable to reduce the bacterial load to decrease mortality at later time points. It has also been reported that L-selectin expression on neutrophils in human, cattle, and rabbit neonates is significantly less than in adults. Blocking L-selectin by use of specific monoclonal antibodies did not significantly affect neutrophil infiltration in neonates. However, monoclonal antibodies against CD18 did significantly reduce infiltration. In lung tissue, both CD18-dependent and -independent mechanisms of adherence are operative. Moreover, in sheep with experimentally induced pulmonary injury, L-selectin is the main target for certain selectin inhibitors, including TBC1269. Therefore, it may be that administration of TBC1269, a selectin inhibitor, in neonates is not sufficient to inhibit neutrophil-mediated damage. Use of both L-selectin and CD18 or β2-integrin antagonists may be required to completely inhibit neutrophil infiltration and subsequent injury in neonatal lungs. Neutrophils are also not the only source of free radicals during endotoxemia. Brigham et al reported that oxidants may be produced by both neutrophils and other cells (eg, endothelial cells). Also, because they have an immature defense system against superoxide radicals, neonates are more susceptible to infections and free radical-induced damage than are adults. Finally, lesions of the lung with less intense inflammation may have significant reduction in CD concentration. Assessment of CD concentration in this study was from the most intense sites of inflammation. This intensity may overwhelm any effects of TBC1269 on CD production.

Apoptosis is a mechanism that can remove cells damaged by reactive oxygen intermediates. Multiple proteolytic proteases (eg, caspases) are involved in apoptosis. The increased number of apoptotic leukocytes (neutrophils and macrophages) in TBC1269-treated calves may potentiate the protective effects of TBC1269 by inhibiting prolonged activation of neutrophils and reducing release of hydrolytic enzymes and severity of tissue damage. Others have shown that apoptosis of neutrophils limits the degree of tissue injury. Macrophage clearance and phagocytosis of apoptotic neutrophils is one mechanism that prevents the release of neutrophil contents into the surrounding tissue.

Nitric oxide is a mediator of many biological functions in the lung. Nitric oxide synthesis is catalyzed by iNOS, which is produced by a variety of pulmonary cells. Nitric oxide production results in tyrosine residue nitration with conversion to peroxynitrite that elicits an inflammatory response. In saline-inoculated calves, the pattern of iNOS expression was consistent with that seen in humans; expression of iNOS is high in airway epithelial cells and chondrocytes. Absorption of our primary antibody with nitrotyrosine was especially useful in confirming staining specificity.

Expression of iNOS in unaffected regions and cells adjacent to the inflammatory areas of lung tissue from M haemolytica-inoculated, untreated and TBC1269-treated calves was similar to that of saline-inoculated calves. Other investigators have demonstrated that several inflammatory stimuli (eg, interferon-γ, LPS) cause various pulmonary cells (eg, alveolar macrophages, epithelial cells, endothelial cells, smooth muscle cells, interstitial cells) to produce iNOS. Pulmonary macrophages and neutrophils also produce iNOS during endotoxin-induced sepsis, and iNOS expression by infiltrating leukocytes may be important for bacterial killing.

To our knowledge, the decrease in iNOS expression by epithelial cells and other parenchymal cells at sites of intense leukocyte infiltration has not been reported. A negative feedback mechanism may explain this finding. Superoxide radicals released from inflammatory cells may inactivate iNOS produced by epithelial cells. This may reduce overall production of NO and other free radicals in lung parenchyma. Although TBC1269 increased susceptibility of leukocytes to apoptosis, it did not appear to alter this feedback mechanism.

The selectin inhibitor TBC1269 enhanced apoptosis during acute M haemolytica pneumonia in calves. Additional studies may more clearly assess the effects of TBC1269 on formation of CD. TBC1269 may prove to be useful clinically for attenuating the detrimental aspects of acute inflammatory processes without entirely eliminating the process. Combined use of TBC1269 with a β2-integrin antagonist may be needed for complete protection against neutrophil-mediated damage.

References


