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Zaher A. Radi
Iowa State University

Karen B. Register
United States Department of Agriculture

E.-K. Lee
United States Department of Agriculture

Marcus E. Kehrli Jr.
United States Department of Agriculture

Kim A. Brogden
United States Department of Agriculture

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What is This?
In Situ Expression of Intercellular Adhesion Molecule-1 (ICAM-1) mRNA in Calves with Acute Pasteurella haemolytica Pneumonia


Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA (ZAR, JMG, MRA); and Avian and Swine Respiratory Diseases Unit (KBR), Metabolic Diseases and Immunology Research Unit (EKL, MEK), and Ruminant and Respiratory Diseases Unit (KAB), USDA-ARS/National Animal Disease Center, Ames, IA

Abstract. The in situ expression of intercellular adhesion molecule-1 (ICAM-1) mRNA in normal and pneumatic lung tissues of Holstein calves with bovine leukocyte adhesion deficiency (BLAD) was compared with that of age-matched non-BLAD Holstein calves by in situ hybridization. Twenty-four Holstein calves (both BLAD and non-BLAD) were randomly assigned to one of two experimental groups and inoculated intrabronchially with Pasteurella haemolytica or pyrogen-free saline. Lung tissues were collected and fixed in 10% neutral formalin at 2 or 4 hours postinoculation (PI). The expression and distribution of ICAM-1 mRNA in the different cell types of the lung tissue was detected by in situ hybridization with a 307-base-pair bovine ICAM-1 riboprobe. In lungs of both non-BLAD and BLAD saline-inoculated calves, ICAM-1 expression was present in epithelial cells but occurred in <30% of cells in bronchi, bronchioles, and alveoli. ICAM-1 expression in vascular endothelial cells was present in >30% of cells in pulmonary arteries and veins. The expression of ICAM-1 was significantly greater (>60% of cells) in bronchiolar and alveolar epithelial cells and pulmonary endothelial cells of arteries and veins in both BLAD and non-BLAD calves inoculated with P. haemolytica. Bronchiolar epithelium had the highest intensity of mRNA expression and highest percentage of cells that were stained, whereas bronchial epithelium had the lowest intensity and percentage of cells stained. Most alveolar macrophages and neutrophils in infected lungs also expressed ICAM-1. ICAM-1 expression was generally increased in infected BLAD calves at 2 hours PI as compared with non-BLAD calves but not at 4 hours PI. The increased expression of ICAM-1 during acute P. haemolytica pneumonia in calves suggests that ICAM-1 is upregulated and may play a role in leukocyte infiltration. The extent of ICAM-1 expression in P. haemolytica-inoculated calves with BLAD was initially enhanced but otherwise similar to that in non-BLAD calves.

Key words: β2-integrins; bovine leukocyte adhesion deficiency; CD18; cattle; in situ hybridization; lung.

Adhesion of circulating leukocytes to the microvascular endothelium is important for their trafficking into sites of inflammation. This interaction depends partially on availability of adhesion molecules expressed by both leukocytes and vascular endothelial cells. Intercellular adhesion molecule-1 (CD54; ICAM-1) is expressed by endothelial cells and is one of the principal ligands for at least two β2-integrin leukocyte surface receptors, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). Binding between β2-integrins and ICAM-1 can mediate passage of neutrophils across the vascular wall, and this infiltration can contribute to endothelial damage and consequent tissue injury. ICAM-1 expression is upregulated in airway epithelium during inflammation and that ICAM-1 is required for inflammatory cell recruitment into the lung of ICAM-1 deficient mice. Alveolar macrophages express a low level of ICAM-1 that increases during acute lung injury. In contrast to these findings in other species, ICAM-1 expression and regulation has not been assessed in the lungs of cattle.

In previous studies of cattle lungs inoculated with Pasteurella haemolytica, initial infiltration into pulmonary bronchi and bronchioles required β2-integrins, whereas alveolar infiltration occurred in the absence of β2-integrin expression. This differential type of infiltration could be related to the degree of expression of ICAM-1. That is, expression of ICAM-1 by specific pulmonary cell types during P. haemolytica infection in cattle may be important for mediating infiltration of activated neutrophils that express β2-integrin. ICAM-
1 expression may be increased in cattle with bovine leukocyte adhesion deficiency (BLAD) because of the absence of \( \beta_2 \)-integrins on neutrophils of BLAD cattle would reduce ICAM-1 binding and, therefore, possibly allow ICAM-1 persistence. Alternatively, increased ICAM-1 expression may be due to enhanced production of proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor alpha (TNF\( \alpha \)) in BLAD cattle or because of a possible lack of production of cytokines that can be inhibitory during inflammation such as IL-4, IL-10, and IL-6. ICAM-1 expression was expected to be higher in bronchi and bronchioles (in comparison with alveoli) in both normal and BLAD calves because of previous findings of \( \beta_2 \)-integrin-dependent infiltration of these airways.\(^1\) To test these hypotheses, the changes in ICAM-1 expression in lung were evaluated after airway instillation of \textit{P. haemolytica} in calves with normal or impaired expression of the \( \beta_2 \)-integrins (CD18) on their leukocytes. Calves with BLAD have impaired expression of \( \beta_2 \)-integrins and have an autosomal recessive genetic mutation that results in <1% expression of the \( \beta_2 \)-integrins.\(^6,17,53\)

**Materials and Methods**

**Experimental animals**

The 24 calves used in this study were raised at the USDA/ARS National Animal Disease Center. The first 12 Holstein calves had deficient expression of \( \beta_2 \)-integrins (CD18) and were diagnosed as homozygous (CD18/−/−) for BLAD allele by polymerase chain reaction (PCR) restriction enzyme analysis as described previously.\(^30,48\) Neutrophils from these calves have impaired function as described by assays in vitro (e.g., random migration, chemiluminescent) and in vivo.\(^3,13,18,39,41\) The second 12 age-matched Holstein calves were heterozygous for the defective CD18 allele but had normal CD18 expression and neutrophil function. These calves were assigned to one of two experimental groups (saline-inoculated control calves or \textit{P. haemolytica}-inoculated calves) and euthanatized 2 or 4 hours postinoculation (PI).

**Inocula**

\textit{Pasteurella haemolytica} was propagated in tryptose broth medium to achieve a culture containing approximately \(1 \times 10^8\) colony-forming units/ml. Each \textit{Pasteurella}-inoculated animal received 5 ml of the \textit{P. haemolytica} broth culture into the right and left cranial lung lobe bronchus using a fiber-optic bronchoscope. Control animals received 5 ml of pyrogen-free saline into each bronchus.

**Tissue collection, fixation, and scoring**

The inoculated animals were euthanatized by intravenous injection with an overdose of sodium pentobarbital immediately prior to tissue collection. Lung tissues were collected from the central area of the inoculation site of each of the 24 animals, which included an area of tissue no less than 2 \( \times \) 2 cm. This region of tissue was subdivided into several smaller sections for tissue sections and for light microscopy. Tissues were fixed in 10% neutral buffered formalin for 24–48 hours. Following fixation, tissues were processed for 48 hours in an automated tissue processor and embedded in paraffin. The intensity of ICAM-1 staining was semiquantitatively measured by assigning a numerical staining score from 0 to 3: 0 = no staining, 1 = minimally detectable staining in <30% of the epithelial cells (airways) or endothelial cells (vessels), 2 = clear moderate staining intensity in 30–60% of the cells, 3 = intense staining in >60% of the cells, and L = special staining designation for intense staining of leukocytes (neutrophils and macrophages). The slides were randomly assigned to two pathologists and scored without previous knowledge of the animal status. The micrographs were selected to best represent the staining intensity.

**Riboprobe preparation**

A DNA fragment from the cDNA bovine ICAM-1 gene was cloned and sequenced. This segment was amplified by PCR using 5′-TGGAGATAGCTGGGCAGGT-3′ (at 1,671 bp) and 5′-GTGGAGCAGTTCAGGAC-3′ primers and subcloned into a pCR\(^{\text{II}}\) plasmid (Invitrogen, Carlsbad, CA).\(^33\) The total length of the cloned segment was 307 bp. Following amplification, the plasmids were isolated, linearized with restriction enzymes \textit{Eco RV} and \textit{Bam HI} (Boehringer Mannheim), and then separated on a 1% agarose gel (Gibco BRL, Gaithersburg, MD) in Tris–acetate–ethylenediaminetetraacetic acid buffer to determine that linearization was complete. Samples of the linearized plasmid were sequenced (DNA Sequencing Facility, Iowa State University, Ames, IA) and compared with the original ICAM-1 sequence to confirm the riboprobe ICAM-1 sequence. The in vitro transcription and labeling of probes were performed using T7-RNA polymerase, SP6-RNA polymerase, and a mixture of nucleotides (ATP, CTP, GTP), including digoxigenin-labeled UTP according to kit instructions (Genius\(^{\text{II}}\) 4 kit, Boehringer Mannheim). Following transcription labeling, the transcription reaction was stopped and the RNA probe was baked on to a nylon membrane and treated sequentially with sheep anti-digoxigenin antibody and alkaline phosphatase (Boehringer Mannheim). The signal was determined by dot blot hybridization using Genius 4 kit components. The probe was compared with serial dilutions of a digoxigenin-labeled RNA strand that were baked on to a nylon membrane and treated sequentially with sheep antidigoxigenin–alkaline phosphatase complex (Boehringer Mannheim) and color substrate solution (nitro blue tetrazolium with 5-bromo-4-chloro-indoyl phosphate). The reaction was then stopped by baking on to a filter and developed with alkaline phosphatase substrate solution (Boehringer Mannheim).

**In situ hybridization protocol**

In situ hybridization procedures were similar to those previously described with few modifications.\(^23,50\) Sections 5 \( \mu \)m thick were cut and placed on silane-coated slides (Fisher Scientific, Pittsburgh, PA). These slides were deparaffinized in two changes (5 minutes each) of xylene and hydrated through graded alcohol baths to diethylpyrocarbonate (DEPC)
(Sigma Chemical Co., St. Louis, MO)-treated water. Slides were then treated with proteinase K (25 μg/ml) (Amresco, Solon, OH) for 30 minutes at 37°C and then rinsed in DEPC water. Sections were incubated with 20% (v/v) cold acetic acid for 40 seconds to inactivate endogenous alkaline phosphatase. The hybridization mixture for each slide was composed of 200 ng of the digoxigenin-labeled ICAM-1 probe added to 48 μl of rapid hybridization buffer (Amersham Life Sciences, Arlington Heights, IL). This mixture was heated at 80°C for 3 minutes, immediately chilled on ice, placed on each section, covered with a glass coverslip, and incubated for 18 hours at 70°C. Following hybridization the coverslips were removed by soaking the slides in 2× standard saline citrate (SSC) twice for 5 minutes each at room temperature. The slides were treated with RNase A (Sigma Chemical Co.) (20 μg/ml) for 30 minutes at room temperature. Slides were subjected to a series of stringency washes in 2× SSC for 5 minutes at 50°C, 1× SSC for 5 minutes at 50°C, 0.5× SSC for 1 hour at 50°C, and 0.5× SSC for 5 minutes at room temperature. The sections were then incubated for 30 minutes at room temperature in 3% blocking reagent (Boehringer Mannheim) and for 2 hours at room temperature with sheep anti-digoxigenin–alkaline phosphatase conjugate diluted 1:300 in Tris-buffered saline (TBS) with 3% normal sheep serum. Sections were rinsed three times (5 minutes each) in TBS. The color substrate solution (nitro blue tetrazolium with 5-bromo-4-chloro-indoly phosphate toluidinium) (Boehringer Mannheim) was then applied to the sections, and slides were incubated in the dark for 20 hours at room temperature. After a final rinse in ultrapure water, the sections were counterstained with nuclear fast red (Sigma Chemical Co.) for 5 minutes and covered with a coverslip. Control sections were either incubated with sense digoxigenin-labeled probe or with hybridization solution with no added probe.

Statistical analysis

Data were analyzed using the chi-square test and Fisher’s exact test. Variables tested consisted of treatments (P. haemolytica or saline), experimental group (BLAD or non-BLAD calves), and pulmonary cell types (bronchial epithelium, bronchiolar epithelium, alveolar epithelium, endothelium of arteries and veins, or neutrophils and macrophages) at 2 or 4 hours PI. The significance level used was 0.05.47

Results

Microscopic lesions

Light microscopic examination revealed infiltrates of neutrophils in airways and alveoli in both BLAD and non-BLAD P. haemolytica-inoculated calves. In non-BLAD animals, neutrophils were present in the bronchiolar lumen, epithelium, lamina propria, and lamina and walls of multifocal bronchi and alveoli. In contrast, BLAD animals had a moderately fewer neutrophils in the bronchioles, bronchi, and alveoli. Also, there were mild multifocal infiltrates of macrophages and mild to moderate hypertrophy of multifocal epithelial cells lining the alveoli.

In situ hybridization

The entire range of staining intensity from 0 to 3 (negative to strong) was present in lung tissues (saline- or P. haemolytica-inoculated calves) (Figs. 1–4). The intensity of ICAM-1 staining on the epithelium and endothelium of saline-inoculated BLAD and non-BLAD animals was negative to minimal (0–1) (Figs. 1, 2). Statistical analysis indicated significant differences in ICAM-1 staining between P. haemolytica and saline groups (P < 0.05) at both 2 and 4 hours PI (Figs. 5–8). In every instance, ICAM-1 staining in P. haemolytica-infected BLAD and non-BLAD animals was significantly (P < 0.05) more intense on the endothelium of pulmonary arteries (A) and veins (V), bronchiolar epithelium (BL), bronchial epithelium (BI), and alveolar epithelium (AL) as compared with staining in uninfected BLAD and non-BLAD saline-inoculated controls (P > 0.05).

BL, AL, and A had the most intense staining in P. haemolytica-inoculated BLAD and non-BLAD calves (Figs. 3, 4). However, staining of BI and V in both groups was less consistent and intense (negative to moderate) in both BLAD and non-BLAD calves inoculated with P. haemolytica. In these two groups, most neutrophils (>90%) and alveolar macrophages that were in airways, alveolar septa, or interstitium had intense staining in all calves. Adjacent vascular smooth muscles had a minimal but identifiable degree of ICAM-1 expression. When data from P. haemolytica-inoculated groups at 2 and 4 hours PI were analyzed, there was a significant increase in ICAM-1 expression (P < 0.05) at 2 hours PI in calves with BLAD as compared with non-BLAD calves (Figs. 5, 6). There was no significant difference between the P. haemolytica-inoculated groups at 4 hours PI (Figs. 7, 8).

Discussion

ICAM-1 is involved in strengthening adhesive interactions between leukocytes and endothelial cells of postcapillary venules and in the infiltration of leukocytes into sites of inflammation.21,26,31,56 The binding of ICAM-1 to integrins mediates passage of neutrophils across the vascular wall. This infiltrative process can contribute to endothelial damage and consequent tissue injury.4,12,13,22,24 In species other than cattle, ICAM-1 is expressed at low levels on resting vascular endothelium and alveolar epithelial cells and by activated monocytes, fibroblasts, and T and B lymphocytes.5,7,9,10,14,42,52 However, ICAM-1 expression and regulation has not been studied in the lungs of cattle.

In this study, mRNA expression of ICAM-1 in lung from both non-BLAD and BLAD saline-inoculated calves was either negative or minimal in the epitheli-
Fig. 1. Lung; control BLAD Holstein calf No. 4, 4 hours PI. Minimal staining (score = 1) ICAM-1 mRNA expression in the pulmonary bronchiolar epithelium (arrows). In situ hybridization technique, nuclear fast red counterstain. Bar = 20 μm.

Fig. 2. Lung; P. haemolytica-inoculated BLAD Holstein calf No. 9, 4 hours PI. Minimal staining (score = 1) ICAM-1 mRNA expression (arrows) in the pulmonary epithelium of alveoli and in the endothelium of pulmonary artery. In situ hybridization technique, nuclear fast red counterstain. Bar = 20 μm.

Fig. 3. Lung; P. haemolytica-inoculated non-BLAD Holstein calf No. 20, 4 hours PI. Intense staining (score = 3) ICAM-1 mRNA expression in the pulmonary bronchiolar epithelium (arrows). The bronchiolar lumen contains several stained neutrophils (arrowhead). In situ hybridization technique, nuclear fast red counterstain. Bar = 20 μm.

Fig. 4. Lung; P. haemolytica-inoculated BLAD Holstein calf No. 9, 4 hours PI. Intense staining (score = 3) ICAM-1 mRNA expression in the endothelium (arrows) of pulmonary artery. In situ hybridization technique, nuclear fast red counterstain. Bar = 40 μm.

um of bronchioles, bronchi, and alveoli and in the endothelium of arteries and veins. There was a minimal but identifiable degree of staining on adjacent vascular smooth muscle cells, which in another study showed little staining for ICAM-1. The significance of this limited amount of ICAM-1 expression in smooth muscle cells is not known.

ICAM-1 expression in the lung is upregulated by proinflammatory cytokines (TNFα and γ-interferon) and after bacterial endotoxin (lipopolysaccharide) exposure. In this study, ICAM-1 was significantly increased in both BLAD and non-BLAD calves after intrabronchial P. haemolytica-induced pulmonary inflammation. ICAM-1 expression was present in the epithelium of bronchioles, bronchi, and alveoli and in the endothelium of arteries and veins. The intensity of staining was significantly increased on the alveolar and bronchiolar epithelium and the endothelium of blood vessels and was slightly increased on the bronchial epithelium after P. haemolytica installation. This finding is consistent with those of previous studies of a murine model for asthma that demonstrated that bronchial epithelial cells have the lowest level of ICAM-1 expression in comparison with other regions of epithelium. Alveolar epithelial cells have higher levels of ICAM-1 expression than the vascular endothelium during pneumonia.

Although ICAM-1 expression was increased in both
BLAD and non-BLAD calves after infection, there was an overall increase in ICAM-1 expression in BLAD animals as compared with non-BLAD calves at 2 hours PI. This increase in expression may indicate an increased transcription level of ICAM-1 in BLAD animals prior to or during the initial stages of infection. BLAD calves may have increased levels of cytokines (TNFα, chemokines) that are produced either locally or systemically. This could be a local compensatory mechanism in the lung resulting from defective neutrophil infiltration. It could also be the result of secondary subclinical infections of other organ systems such as the alimentary or urinary tracts.

The increased ICAM-1 expression in airway epithelium may facilitate cell injury by increasing leukocyte (neutrophil) adhesion in acute *P. haemolytica* pneumonia. The increased ICAM-1 expression by these cells during infection agrees with findings in previous studies using other experimental animal models of lung diseases. Increased ICAM-1 expression has been found in lung during acute inflammatory response in murine and canine models. These studies, however, did not specify the degree of ICAM-1 expression and distribution within the different pulmonary cell types. Therefore, this aspect of ICAM-1 expression cannot be compared directly to the results of this study. Other investigators have demonstrated that ICAM-1 is present at a basal level in both alveolar epithelium and vascular pulmonary endothelium but constitutively expressed following endotoxin inoculation by vascular endothelial cells.

The present finding that neutrophils and macrophages expressed ICAM-1 in response to infection is consistent with other findings demonstrating that human neutrophils and alveolar macrophages express ICAM-1. The importance of ICAM-1 in pneumonia and neutrophil infiltration has been demonstrated. However, the type of inoculum is an important factor for neutrophil margination. *Escherichia coli* induces a CD18-dependent type of neutrophil migration, whereas *Streptococcus* induces CD18-independent type of migration. In a rabbit model, a CD18-dependent mechanism in acute lung inflammation and a CD18-independent mechanism in chronic inflammation was found.

Although β₂-integrins are important for leukocyte transmigration, it was previously reported that a
CD18-independent mechanism for neutrophil migration can occur in acute lung injury. A CD18-independent mechanism was also demonstrated for neutrophil infiltration in BLAD cattle into the alveolar space. Although neutrophils of BLAD animals lack β2-integrins, tissues in this study had numerous neutrophils in the alveolar lumina, consistent with previous results. Alveolar macrophages may release factors that are important for neutrophil infiltration in the alveolar space via a non-CD18 mechanism.

ICAM-1 upregulation on bronchiolar epithelium and pulmonary vascular endothelium may facilitate recruitment and adhesion of leukocytes to these cells and may play an important role in neutrophil infiltration and consequent tissue damage in bovine pulmonary pasteurellosis. Further in vivo studies of this ICAM-1-mediated leukocyte–airway interaction are necessary to better understand the pathophysiology and possible therapeutic consequences in acute bovine *P. haemolytica* pneumonia.

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Request reprints from Dr. Z. A. Radi, Department of Veterinary Pathology, 2740 Veterinary Medicine, Iowa State University, Ames, IA 50011-1250 (USA). E-mail: radi@iastate.edu.