The airway epithelium plays a critical role in maintaining mucosal integrity and in providing a physical barrier to inhaled noxious stimuli and infectious agents. By virtue of its location, ability to express CSRs, and capacity to produce and release cytokines and other proteins, the airway epithelium exerts a major influence in the development of pulmonary immune and inflammatory responses. For example, in bronchial tissues obtained from humans with chronic obstructive pulmonary disease or severe asthma, there is an upregulation of epithelial-derived chemokines, growth factors, and CSRs that is thought to play a major role in the pathophysiologic mechanisms of disease. Furthermore, these differences in the expression of selected proteins (eg, cytokines and receptors) are often evident in BECCs established from individuals with obstructive lung diseases.

**Effects of in vitro exposure to hay dust on the gene expression of chemokines and cell-surface receptors in primary bronchial epithelial cell cultures established from horses with chronic recurrent airway obstruction**

Dorothy M. Ainsworth, DVM, PhD; MaryBeth Matychak, BSc; Claudia L. Reyner, BSc; Hollis N. Erb, DVM, PhD; Jean C. Young, BSc

**Objective**—To examine effects of in vitro exposure to solutions of hay dust, lipopolysaccharide (LPS), or β-glucan on chemokine and cell-surface receptor (CSR) gene expression in primary bronchial epithelial cell cultures (BECCs) established from healthy horses and horses with recurrent airway obstruction (RAO).

**Sample Population**—BECCs established from bronchial biopsy specimens of 6 RAO-affected horses and 6 healthy horses.

**Procedures**—5-day-old BECCs were treated with PBS solution, hay dust solutions, LPS, or β-glucan for 6 or 24 hours. Gene expression of interleukin (IL)-8, chemokine (C-X-C motif) ligand 2 (CXCL2), IL-1β, toll-like receptor 2, toll-like receptor 4, IL-1 receptor 1, and glyceraldehyde 3-phosphate dehydrogenase was measured with a kinetic PCR assay.

**Results**—Treatment with PBS solution for 6 or 24 hours was not associated with a significant difference in chemokine or CSR expression between BECCs from either group of horses. In all BECCs, treatment with hay dust or LPS for 6 hours increased IL-8, CXCL2, and IL-1β gene expression >3-fold; at 24 hours, only IL-1β expression was upregulated by >3-fold. In all BECCs, CSR gene expression was not increased following any treatment. With the exception of a 3.7-fold upregulation of CXCL2 in BECCs from RAO-affected horses (following 6-hour hay dust treatment), no differences in chemokine or CSR gene expression were detected between the 2 groups. At 24 hours, CXCL2 gene expression in all BECCs was downregulated.

**Conclusions and Clinical Relevance**—Epithelial CXCL2 upregulation in response to hay dust particulates may incite early airway neutrophilia in horses with RAO. (Am J Vet Res 2009;70:365–372)

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Received April 29, 2008. Accepted June 25, 2008.

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Supported by USDA National Competitive Research Grant No. 2004-01233.

The authors thank Carol Collyer for technical assistance. Address correspondence to Dr. Ainsworth.

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BECC</td>
<td>Bronchial epithelial cell culture</td>
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<tr>
<td>CSR</td>
<td>Cell-surface receptor</td>
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<tr>
<td>CT</td>
<td>Threshold cycle number</td>
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<tr>
<td>ΔCT</td>
<td>Standardized threshold cycle number for the target gene</td>
</tr>
<tr>
<td>ΔPpl max</td>
<td>Maximal change in pleural pressure during tidal breathing</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>RAO</td>
<td>Recurrent airway obstruction</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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The airway epithelium plays a critical role in maintaining mucosal integrity and in providing a physical barrier to inhaled noxious stimuli and infectious agents. By virtue of its location, ability to express CSRs, and capacity to produce and release cytokines and other proteins, the airway epithelium exerts a major influence in the development of pulmonary immune and inflammatory responses. For example, in bronchial tissues obtained from humans with chronic obstructive pulmonary disease or severe asthma, there is an upregulation of epithelial-derived chemokines, growth factors, and CSRs that is thought to play a major role in the pathophysiologic mechanisms of disease. Furthermore, these differences in the expression of selected proteins (eg, cytokines and receptors) are often evident in BECCs established from individuals with obstructive lung diseases.
Not surprisingly, horses with RAO also have up-regulation of transcription factors or chemokines in the airway epithelium following inhalation of organic particulates that are thought to contribute to the inflammatory reaction. However, it is currently unknown whether such differences remain apparent in primary BECCs from RAO-affected horses. Thus, the purpose of the study reported here was to determine whether chemokine and CSR gene expression in primary BECCs established from horses chronically affected with RAO is different from that of control horses. Gene expressions of IL-8, CXCL2, and IL-1β were examined because these proteins are potent neutrophil chemoattractants and hay dust–induced alterations in their expression in vitro might provide supportive evidence for their roles in the development of pulmonary neutrophilia in vivo. The cell-surface gene expression of TLR2, TLR4, and IL-1 receptor 1 was studied because changes in the gene expression of these receptors in vitro, as a function of ligand exposure, suggest involvement of that receptor. Furthermore, in murine airway epithelial cells, Aspergillus fumigatus and endotoxin (components of hay dust) signal through TLR4 and LPS-induced pulmonary inflammation requires participation of the IL-1 receptor 1.

**Materials and Methods**

**Animals**—Primary BECCs were established from the lung samples obtained from 6 RAO-affected horses and 6 control horses. The RAO-affected group consisted of 1 mare and 5 geldings (455 to 550 kg) that had a median age of 19 (range, 15 to 21) years. The group comprised 4 Quarter Horses, 1 Arabian, and 1 Paso Fino horse that consistently developed RAO when fed dusty hay while housed in a stable bedded with shavings. Criteria used to define the RAO phenotype were the development of pulmonary neutrophilia (≥25% neutrophils in the BALF) and an accentuated breathing effort (∆Ppl max ≥ 15 cm H2O). The control group consisted of 3 mares and 3 geldings (300 to 357 kg) that had a median age of 17 (range, 4 to 27) years. This group comprised 4 Thoroughbreds, 1 warmblood, and 1 Quarter Horse. Control horses did not develop pulmonary neutrophilia or an increase in ∆Ppl max when housed in a stable and fed dusty hay.

On the basis of findings on physical, endoscopic, and thoracic radiographic examinations, all horses had been confirmed to be healthy prior to the start of the experiment. Several months previously, all horses were dewormed by administration of ivermectin and vaccinated against tetanus, influenza, eastern equine encephalomyelitis, western equine encephalomyelitis, and rabies. All experimental procedures were approved by the Animal Care Committee of Cornell University and were in accordance with guidelines established by the National Institutes of Health.

**Experimental design**—Prior to the start of the experiment, all horses had been maintained on pasture for 2 to 3 months so that RAO-susceptible horses were free of clinical signs. Both groups of horses were moved to individual box stalls (4 × 4 m) that were bedded with wood shavings and were provided unlimited access to water and dusty timothy-alfalfa hay ad libitum for the 2-week study period. The period of 2 weeks was chosen to ensure development of the RAO phenotype and to induce upregulation of chemokine gene expression in the airway epithelium of the diseased horses.

On day 13 of the study, ∆Ppl max values were measured in unsedated horses by use of an esophageal balloon catheter connected to a pressure transducer. In each horse, the mean ∆Ppl max was obtained for 10 to 15 breaths. Horses were then sedated by administration of xylazine hydrochloride (0.6 mg/kg, IV), and a 2.1-m videolaryngoscope was passed via the nasal passages into the distal portion of the trachea. Following local anesthesia of the airways by infusion of 25 mL of 2% lidocaine hydrochloride, the endoscope was wedged in a sixth- to ninth-generation bronchiole of the right lung lobe. A bronchoalveolar lavage was performed by instilling 300 mL of warm (37°C) sterile saline (0.9% NaCl) solution through the biopsy channel followed immediately by gentle aspiration. The BALF samples were stored in siliconized containers that were placed on ice until transported to the laboratory. Horses were then euthanized by IV administration of pentobarbital and phenytoin (95 mg/kg), and the lungs were aseptically removed within 50 minutes through a sterile thoracotomy that included resection of the 9th to 11th ribs. The left and right lung lobes were rinsed with sterile saline solution, placed in a sterile container, and immediately transported to the laboratory for processing in a laminar-flow hood.

**Preparation of samples**—The BALF cells were processed for cytologic analysis by mixing 900 µL of BALF with 100 µL of 1% osmethylthreitol. Samples that underwent cytocentrifugation were stained, and a differential cell count was performed by examination of 200 cells.

The bronchi (2nd- to 15th-generation airways) were removed from the lungs by means of a sterile technique, transected into 3- to 5-cm pieces, and washed 3 times with PBS solution containing penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL). Four airway segments were then placed in 50-mL tubes that contained 30 mL of PBS solution with 0.1% protease I and 0.001% DNAse. Tubes were incubated on a rocking platform at 4°C for 24 to 48 hours. At the conclusion of the incubation period, 3 mL of fetal bovine serum was added (final concentration, 10%) to inactivate the enzymes. Bronchial segments were transferred to a culture dish containing PBS solution with 10% fetal bovine serum so that the epithelial cells could be gently dislodged with a scalpel blade. Cells were centrifuged at 200 X g for 10 minutes (4°C), washed twice, and resuspended (2 × 10⁶ cells/mL) in complete medium. One milliliter of the cell suspension was used to seed each 2-cm² well that previously had been coated overnight and dried with 500 µL of a coating solution. The latter consisted of 0.01% bovine serum albumin, 0.003% collagen, and 0.001% fibronectin in medium. Cells were incubated at 37°C in humidified air with 3% CO₂ until 80% confluency was achieved (on day 3). Until confluency was achieved, culture medium was replaced every 48 hours. Preliminary immunohistochemistry data of cell cultures stained for
cytokeratin, an epithelial-cell marker, revealed homogeneity of the cell cultures by day 5 (Figure 1). The supernatant was removed from each cell culture well, cells were gently washed, and the complete medium was replaced. Each well received 1 of 5 treatments as follows: 100 µL of PBS, 100 µL of a hay dust solution (2 different log concentrations, description to follow), 100 µL of LPS solution with a final concentration of 10 µg LPS/mL, and 100 µL of a β-glucan solution with a final concentration of 12.5 ng β-glucan/mL. The PBS solution and hay dust treatments were included to determine whether an inherent upregulation existed in BECCs from RAO-affected horses (PBS solution treatment) or whether the cell cultures required continuous exposure to hay dust solution to manifest differences in chemokine or CSR expression (hay dust). Cells were incubated for 6 or 24 hours (both times for each treatment) at 37°C in humidified air with 5% CO₂. At the conclusion of the incubation period, the supernatant was removed and 600 µL of lysis buffer was added to each well. Contents from each well were frozen at -80°C until gene expression studies were conducted.

The hay dust was prepared as previously described. Briefly, flakes of dusty hay were agitated onto a clean surface, sieved through a grid (2 × 3 mm), and separated into fine from coarse particles by use of a dual vortex vacuum cleaner. Ten milliliters of sterile saline solution was added to each gram of hay dust, and the solution was filtered through a mesh screen (100-µm pore diameter). The hay dust solution was then γ-irradiated (1.5 Mrad for 12 hours, with samples maintained on dry ice) to sterilize the solution and to prevent bacterial and fungal overgrowth of the cell cultures. Particulates in the stock solution were counted with a hemocytometer, and endotoxin and β-glucan concentrations were measured at a commercial laboratory. Each milliliter of the stock solution of hay dust contained 10⁸ fungal particulates, endotoxin at 2 µg/mL, and β-glucan at 12.5 ng/mL. Each well that was treated with hay dust solution 1 contained 10⁷ fungal particulates, 2 ng of endotoxin, and 12.5 pg of β-glucan. Each well that was treated with hay dust solution 2 contained 10⁷ fungal particulates, 0.2 ng of endotoxin, and 1.3 pg of β-glucan.

**Measurement of gene expression** — Total RNA was extracted, genomic DNA was destroyed, and cDNA was synthesized. Gene expression was measured by use of a real-time reverse transcriptase–PCR assay. Target genes of interest were the chemokines IL-8, CXCL2, and IL-1β and the epithelial CSRs TLR2, TLR4, and IL-1 receptor 1. Target gene expression was standardized to the housekeeping gene GAPDH (Appendix). The PCR reaction mixtures had a final volume of 27.5 µL (2.5 µL of cDNA and 25 µL of the master mix). For each cDNA sample, triplicate reactions were performed.

<table>
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<th>HDS 1 24 hours</th>
<th>HDS 2 6 hours</th>
<th>HDS 2 24 hours</th>
<th>LPS (10 µg) 6 hours</th>
<th>LPS (10 µg) 24 hours</th>
<th>Glucan (12.5 ng) 6 hours</th>
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<td>1.0</td>
<td>1.1</td>
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*Indicates downregulation, compared with treatment with PBS solution. HDS = Hay dust solution. IL-1R1 = IL-1 receptor 1.

Figure 1 — Photomicrograph of primary epithelial cell cultures (6 days old) that were established from an RAO-affected horse. Cells were stained for cytokeratin (red-orange), a marker for epithelial cells, and counter stained with hematoxylin; bar = 50 µm.
on each plate for detection of target genes. Negative control samples (lacking cDNA) and positive control samples (cDNA from LPS-stimulated monocytes) were included on each plate.

The end point used in the real-time reverse transcriptase–PCR quantification was the Ct, at which the amplicon was detected. The Ct ranged from 0 to 40. Gene expression was reported as the ΔCt or as the fold change in the target gene.21 For the first method, ΔCt was the difference between the Ct of the target gene and the Ct of the housekeeping gene, GAPDH. In general, the smaller the ΔCt value, the more cDNA (ie, mRNA) was contained in a sample. For the second method, the fold change was calculated as 2^{-ΔΔCt}. The ΔΔCt represented the difference between the ΔCt for a specific treatment (eg, LPS-treated cells) of BECCs from control horses relative to the ΔCt for that specific treatment of BECCs from RAO-affected horses.

Statistical analysis—For a given treatment, differences in the pulmonary function variables between the 2 groups of horses and in the ΔCt between BECCs established from the 2 groups of horses were detected by use of the Wilcoxon rank sum test. Because of the multiple comparisons that were made, a Bonferroni correction was applied. This was calculated by dividing alpha (0.05) by the number of genes to be compared. Thus, for evaluation of either the chemokine or CSR responses, P = 0.05/3 = 0.017 was used to indicate significance. All computations were performed by use of a statistical software program.8

Results

Pulmonary function and cellular composition of BALF—After 2 weeks of stabling and exposure to dusty hay, RAO-susceptible horses (but not control horses) developed signs of RAO. The mean ± SD ∆Ppl max of the RAO-susceptible horses (34 ± 19 cm H₂O) was significantly (P = 0.001) greater than that of control horses (6 ± 1 cm H₂O). Mean percentage of neutrophils in BALF of diseased horses (52 ± 18%) was significantly (P < 0.001) greater than that of control horses (6 ± 5%).

Effect of treatment on chemokine gene expression of primary BECCs—Within-group treatment–associated changes in chemokine expression relative to chemokine expression in PBS solution were determined (Table 1). For BECCs established from either group of horses, the 6-hour incubation with the solutions of hay dust or LPS was associated with a >3-fold increase in IL-8, CXCL2, or IL-1β gene expression. When cells were incubated for 24 hours with solutions of hay dust or LPS, only the IL-1β gene expression was increased ≥3-fold relative to IL-1β gene expression in control PBS solution.

Box-and-whisker plots were created of standardized Ct values for IL-8, CXCL2, and IL-1β after incubation with each of the 5 treatments (Figure 2). After treatment with PBS solution for 6 or 24 hours, gene expression of the 3 chemokines did not differ significantly between BECCs established from the 2 groups of horses. With the exception of early CXCL2 gene expression, the response of the primary epithelial cells isolated from RAO-affected horses was not different from that of control horses following treatments with the hay dust, LPS, or β-glucan solutions for 6 or 24 hours. Following 6-hour incubation with hay dust solution 1, CXCL2 gene expression was significantly (3.7-fold) greater in BECCs from RAO-affected horses,
compared with BECCs established from control horses. A similar change in CXCL2 upregulation (2.5-fold) was also observed for BECCs established from cells isolated from RAO-affected horses treated with hay dust solution 2, but this difference was not significant ($P = 0.04$). A difference in CXCL2 gene expression between BECCs established from the 2 groups of horses was not apparent following the 24-hour incubation with the 2 hay dust solutions. However, the median $\Delta C_T$ of CXCL2 for 6-hour treatments increased (ie, mRNA expression decreased), compared with the median $C_T$ for 6-hour treatments. This downregulation, unique to CXCL2, was not attributed to a change in the gene expression of GAPDH because the median $C_T$ of GAPDH for the entire group of horses in the 6-hour treatments (20.2) was not significantly different from that in the 24-hour treatments (19.9).

**Effect on CSR gene expression of primary BECCs**—After treatment with PBS solution for 6 or 24 hours, a significant difference was not found in gene expression of TLR2, TLR4, or IL-1 receptor 1 between BECCs established from the 2 groups of horses. In BECCs established from either control horses or RAO-affected horses, TLR2, TLR4, or IL-1 receptor 1 gene expression was not increased >2-fold relative to responses to control PBS solution (Table 1).

Box-and-whisker plots were created of standardized $C_T$ values for TLR2, TLR4, and IL-1 receptor 1 after incubation with each of the 5 treatments (Figure 3). For all of the 4 ligand treatments for 6 or 24 hours, no significant differences in TLR2, TLR4, or IL-1 receptor 1 gene expression were found between BECCs established from the 2 groups of horses.

**Discussion**

The use of primary bronchial cell cultures to investigate respiratory disorders of horses is not unique to this study—others have used similar culture systems to investigate viral pathogens of horses. We are, however, unaware of other studies that have established primary BECCs from RAO-affected horses to elucidate inherent or treatment-induced differences in epithelial chemokine and CSR gene expression. Primary BECC systems offer a unique advantage over studies in animals in that the effects of multiple ligands administered for varying treatment times can be systematically and simultaneously examined in homogenous cell populations.

Our data indicate that following treatment with PBS solution for either 6 or 24 hours, no inherent differences in the chemokine (IL-8, CXCL2, or IL-1β) or in CSR (TLR2, TLR4, or IL-1 receptor 1) gene expression exist between the BECCs established from healthy and RAO-affected horses. Second, exposure of BECCs to solutions of hay dust or LPS for as little as 6 hours was associated with a >3-fold increase in chemokine gene expression. Third, in contrast to chemokine gene expression, the hay dust or component treatment failed to upregulate TLR2, TLR4, or IL-1 receptor 1 gene expression in BECCs from either group of horses regardless of the treatment time. Fourth, the magnitude of the treatment responses of BECCs from RAO-affected horses was not significantly different from that of BECCs from control horses, with the single exception of CXCL2 gene expression. Short-term hay dust exposure upregulated CXCL2 gene expression nearly 4-fold in BECCs from diseased horses, compared with that of BECCs from control horses. However, even though the 24-hour treatment with hay dust (or LPS) was not associated with a differential gene expression in CXCL2 between BECCs from the 2 groups of horses, the median CXCL2 gene expression for all 24-hour treatments (in both groups) was downregulated.
compared with the median responses for the 6-hour treatments.

When RAO-susceptible horses are stabled and exposed to dusty hay for as little as 5 hours, significant increases in airway neutrophil percentages and in breathing flow (Ppl max) occur. Control horses, exposed to similar environmental challenges, fail to develop these same alterations. Mechanisms inciting the neutrophil influx or the alterations in pulmonary function tests remain uncertain. However, the lung represents the largest epithelial surface in the body; the respiratory tract epithelium is the first tissue to interact with airborne dusts and particulates, and the epithelium responds to various particulates and allergens by the release of effector molecules such as chemokines, cytokines, antimicrobial peptides, and adhesion molecules. Therefore, it is logical to investigate the role of this tissue in inciting the early neutrophil influx in horses with RAO.

To date, over 50 chemokines (encompassing 4 structural families and interacting with numerous CSRs) have been identified. These chemotactic polypeptides are classified on the basis of the location of conserved cysteine motifs in the protein structure and are designated as C, C-C, C-X-C, and CX3C chemokines. In inflammatory conditions, the C-X-C chemokines are the most critical for neutrophil recruitment and are represented by CXCL1 (formerly called growth-related oncogene–α, macrophage inflammatory protein–1α, and melanoma growth stimulatory activity–α), CXCL2 (also known as growth-related oncogene–β, macrophage inflammatory protein–2, and melanoma growth stimulatory activity–β), and CXCL8 (still most frequently referred to as IL-8). In mice with experimentally induced asthma, global transcript-expression profiling has been used to define the spectrum and kinetics of chemokine involvement following inhaled allergen exposure and to gain an understanding of the pathophysiologic mechanisms of the disorder. For example, within 3 hours of allergen inhalation, gene expression of CXCL1 and CXCL2 in the lungs is immediately upregulated. Although CXCL1 gene expression remains increased for at least an additional 18 hours, CXCL2 gene expression rapidly decreases during this time. In addition to the effects of inhaled allergen or particulates, epithelial chemokine transcription is also induced by cytokines such as IL-1β. Within 4 hours of intratracheal instillation of IL-1β into rodent lungs, there is an increased gene expression of CXCL2 in the bronchial epithelium and this increase coincides with neutrophil influx into the lungs. However, within 24 hours of the IL-1β installation, the enhanced CXCL2 gene expression in the airway epithelium has disappeared.

In bronchial biopsy specimens obtained from RAO-affected horses, differential gene expression of CXCL1, CXCL2, IL-8, and tumor necrosis factor–α as a function of disease development or treatment strategies has also been investigated. Because significant differences in epithelial CXCL1 or in tumor necrosis factor–α gene expression fail to develop in susceptible horses following hay dust exposure, it has been suggested that these epithelial-derived cytokines play a minor role in the development of airway neutrophilia. Furthermore, even though significant increases in bronchial epithelial IL-8 mRNA and protein concentrations develop in RAO-affected horses, these chemokine changes are not apparent until at least 24 hours after continuous dusty hay exposure. These findings also suggest that epithelial-derived IL-8 does not incite the early neutrophil influx. Although it is tempting to postulate (on the basis of our in vitro results) that the initial granulocyte influx in vivo is incited by CXCL2 synthesis and release by airway epithelium in response to inhaled hay dust particulates, appropriate longitudinal studies remain to be conducted. Additionally, gene expression studies also need to be confirmed by appropriate measurements of CXCL2 protein concentrations in the airway epithelium. Nevertheless, it is noteworthy that CXCL2 expression kinetics in equine primary BECCs following hay dust exposure (rapid upregulation by 6 hours and subsequent downregulation by 24 hours) in our study share similarities with the in vivo gene expression kinetics of CXCL2 in rodents following allergen inhalation.

Of the 3 chemokines examined in the current study, the IL-1β gene expression was most markedly upregulated (compared with IL-1β gene expression in response PBS solution) following hay dust or LPS treatment. The experimental design of the current investigation did not permit partitioning out of the modulating role that IL-1β might have on CXCL2 gene expression in vitro, similar to what occurs in rodents in vivo. Moreover, gene expression, protein release, and control of IL-1β bioactivity are complex. Bioactivity of IL-1β is blocked by an IL-1 receptor antagonist that binds to the IL-1 receptor type 1. Occupation of the receptor by the IL-1 receptor antagonist does not result in intracellular signal transduction and subsequent gene transcription. Bioactivity of IL-1β is also attenuated by a membrane-bound decoy receptor, IL-1 receptor type 2, that can be shed by activated cells and reversibly bind IL-1β. It is possible, therefore, that despite the lack of differential expression of either IL-1β or IL-1 receptor 1 gene expression in equine primary BECCs following treatment with PBS solution, hay dust, or LPS, differences in the gene expression and release of IL-1β inhibitors (IL-1 receptor antagonist or IL-1 receptor type 2) existed. An increase in the effective IL-1β concentrations would have enhanced early CXCL2 expression in the bronchial epithelial cells of RAO-affected horses.

The bronchial epithelium contains a plethora of CSRs that respond to inhaled particulates and cell-released cytokines. These epithelial receptors include but are not limited to the TLRs, mannose receptors, and protease-activated receptors as well as receptors for tumor necrosis factor–α, IL-1β, epidermal growth factor, IL-4, IL-13, IL-17, and interferon-γ. Of particular interest is the potential role of TLRs in RAO because involvement of this family of proteins has been implicated or proven in a variety of inflammatory lung diseases such as chronic obstructive pulmonary disease, asthma, and bronchiolitis. Activation of TLR4 by LPS or TLR2 by its ligands (lipopolysaccharide, zymosan, and peptidoglycan) eventually leads to the nuclear translocation of activated nuclear factor–κB and subsequent transcription of cytokines, chemokines, hematopoietic factors, acute-phase proteins, and antimicrobial factors.
ment of TLRs often leads to changes in their own gene expression in that cell. For example, primary human airway epithelial cell cultures constitutively express mRNA for TLR2 and TLR4, which is modestly upregulated (≤ 2-fold) following treatment with zymosan and LPS, respectively. However, significant upregulation of TLR4 expression in primary human airway epithelial cell cultures exposed to LPS occurs when epithelial cells are also cocultured with a small number of monocytes. Perhaps then, the lack of significant treatment-induced increases in TLR2 or TLR4 gene expression in equine primary BECCs reflects the absence of monocyte-derived factors needed for enhanced transcription of these CSRs. Yet, our in vitro results confirm a previous report\(^1\) from our laboratory that revealed no difference in TLR4 gene expression in bronchial epithelial biopsy specimens obtained from RAO-affected horses, compared with control horses. It is also possible that the increased TLR4 gene expression that Berndt et al\(^2\) reported in bronchial brushing samples obtained from RAO-affected horses relative to control horses reflected the potential contributions of airway mononuclear cells that were included in bronchial brushing samples.\(^9\)

In conclusion, inherent differences in the gene expression of IL-1β, CXCL2, and IL-8 or in the CSRs TLR2, TLR4, or IL-1 receptor 1 were not apparent in primary BECCs established from RAO-affected and healthy horses. The unique kinetic gene expression profile of CXCL2 that developed in BECCs from RAO-affected horses following hay dust exposure might be clinically relevant and contribute to the early neutrophil influx in horses with RAO. Elucidation of the mechanistic pathways by which enhanced transcription of CXCL2 occurs in RAO-affected horses awaits additional studies.

References


a. Equalan, Merial Ltd, Iselin, NJ
b. Encevac TC-4 with Havlogen, Intervet Inc, Millsboro, Del.
c. Imrab, Merial Ltd, Iselin, NJ.
d. Tranquived, Vedco Inc, St Joseph, Mo.
e. Olympus CV-100 videendoscope, Olympus of America, Melville, NY.
f. Lidocaine 2%, Butler Co, Columbus, Ohio.
g. Sodium chloride solution, Baxter, Deerfield, Ill.
i. Sigma-Aldrich Co, St Louis, Mo.
k. Antibiotic/Anitmycotic 100X, Invitrogen, Grand Island, NY.
l. Protease Type XIV, Sigma-Aldrich Co, St Louis, Mo.
m. Sigma-Aldrich Co, St Louis, Mo.

References

mRNA expression in bronchial tissues compared to bronchoalveolar lavage cells in horses with recurrent airway obstruction.


31. Miao XQ, Kawai M, Yamashita T, et al. Imbalance production between interleukin-1β (IL-1β) and IL-1 receptor antagonist (IL-1Ra) in bronchial asthma. *Biochem Biophys Res Commun* 2000;276:607–612.


### Appendix

**Primer and probe sequences used in real-time reverse transcriptase–PCR assays.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession No.</th>
<th>Forward primer (5′ to 3′)</th>
<th>Reverse primer (5′ to 3′)</th>
<th>Probe (5′ to 3′)</th>
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