Inflammatory airway disease is a performance-limiting condition of athletic horses and is characterized by alterations in pulmonary function, such as airway hyperresponsiveness, expiratory flow limitation, and impaired gas exchange during exercise. Analysis of BAL fluid samples obtained from horses with IAD typically reveals abnormal cytologic profiles; the abnormalities commonly include high total nucleated cell counts, mild neutrophilia, lymphocytosis, and monocytosis. Some horses with IAD also have higher than normal numbers of eosinophils or mast cells in BAL fluid samples, reflecting phenotypic variations of IAD.

The etiopathogenesis of IAD in horses remains poorly understood and probably is multifactorial. An etiologic role of infection (bacterial or viral) has been advocated by some investigators on the basis of findings in affected horses such as bacterial isolates in tracheal aspirates, high titers of serum antibodies against equine herpesvirus 1, or resolution of pulmonary inflammation induced by influenza virus or respiratory syncytial virus.

Effects of in vitro exposure to autologous blood and serum on expression of interleukin-8, interleukin-1β, and chemokine (C-X-C motif) ligand 2 in equine primary bronchial epithelial cell cultures

Dorothy M. Ainsworth, DVM, PhD, and Claudia L. Reyner, BS

Objective—To examine the effects of in vitro exposure to solutions of autologous horse blood (AHB) and autologous horse serum (AHS) on expressions of selected cytokine genes in equine primary bronchial epithelial cell (BEC) cultures and to contrast these responses to those induced in BEC cultures by endotoxin and hay dust.

Sample—BEC cultures established from bronchi of 6 healthy horses.

Procedures—5-day-old BEC cultures were treated with PBS solution, AHB (2 concentrations), AHS, hay dust solution, and lipopolysaccharide solution for 24 hours. Gene expressions of interleukin (IL)-8, IL-1β, chemokine (C-X-C motif) ligand 2 (CXCL2), and glyceraldehyde-3-phosphate dehydrogenase were subsequently measured with a kinetic PCR assay.

Results—With the exception of AHS, all treatments of the BECs resulted in upregulation of each target gene expression relative to its expression in cultures exposed to PBS solution. Treatment with AHB induced a dose-dependent increase of each target gene, with IL-1β expression increasing the most (>1,200-fold increase). Lipopolysaccharide and hay dust solution treatments each resulted in 20-fold increases in IL-8 and IL-1β gene expressions. Lipopolysaccharide and hay dust solution treatments also resulted in a 7- and 8-fold increase in CXCL2 gene expression, respectively. The increases in IL-8 and CXCL2 gene expressions following treatment with the higher concentration of blood were equivalent to those associated with hay dust solution or lipopolysaccharide.

Conclusions and Clinical Relevance—Results suggested that chemokine expression by cultured equine BECs following exposure to pulmonary hemorrhage conditions may contribute to the development of inflammatory airway disease in horses. (Am J Vet Res 2012;73:296–301)
following oral treatment with interferon-α. Environmental particulates such as organic dust, endotoxin, and β-glucan have been implicated in the development of IAD because stimulating promotes airway inflammation characterized by increases in IL-6 gene expression in BAL fluid cells and neutrophilia in BAL fluid. Finally, exercise-induced pulmonary hemorrhage has also been suggested to be a cause of IAD in athletic horses. This is based on the findings that the numbers of total nucleated cells, neutrophils, and hemosiderophages in BAL fluid samples obtained from racehorses increase as a function of age, that neutrophilia and erythrocytosis in BAL fluid increase in racehorses as exercise intensity increases; and that alterations in pulmonary function tests or in differential cell counts in BAL fluid occur following instillation of AHB in the lungs of horses. However, a correlation between BAL fluid hemosiderophage or neutrophil counts in horses with IAD has not been universally found by other investigators.

Although a causal relationship between pulmonary hemorrhage and IAD may exist, it remains to be established what components of blood (cellular or soluble) or what pulmonary cells (luminal, interstitial, or bronchial) initiate the inflammatory response. The chemokines IL-8, IL-1β, and CXCL2 are inflammatory mediators; their expressions are upregulated in horses with recurrent airway obstruction, and they could potentially contribute to bronchial influx of neutrophils in horses with IAD. The biological actions of these 3 cytokines have previously been reviewed. Interleukin-8, a potent chemoattractant for neutrophils both in vitro and in vivo, is produced by numerous cells, including alveolar macrophages, monocytes, neutrophils, BECs, and pulmonary endothelial cells. Interleukin-8 transcription is regulated by nuclear factor κB and intranuclear activator protein-1. Interleukin-8 and CXCL2 exert their chemotactic effects on neutrophils by engaging the cell surface receptor, CXCR2, with subsequent activation of cytoskeletal reorganization and cell chemotaxis. In rodents (which lack IL-8), the chemoattractant properties of CXCL2 (formerly known as macrophage inflammatory protein-2) belong to the C-X-C chemokine family, the members of which have conserved cysteine motifs at the amino terminal end. Interleukin-8, a potent chemoattractant for neutrophils both in vitro and in vivo, is produced by numerous cells, including alveolar macrophages, monocytes, neutrophils, BECs, and pulmonary endothelial cells. Interleukin-8 transcription is regulated by nuclear factor κB and intranuclear activator protein-1. Interleukin-8 and CXCL2 exert their chemotactic effects on neutrophils by engaging the cell surface receptor, CXCR2, with subsequent activation of cytoskeletal reorganization and cell chemotaxis. In rodents (which lack IL-8), the chemoattractant properties of CXCL2 have been investigated in vivo in models of asthma. Following allergen or particulate exposure, there is a rapid upregulation of CXCL2 in rodent lungs followed by the development of pulmonary neutrophilia. Interleukin-1β exerts its chemotactic properties primarily by inducing nuclear translocation of nuclear factor κB and secondary transcription of IL-8 and CXCL2. Within 4 hours after intratracheal instillation of IL-1β in rodents, there is an increase in bronchial epithelial CXCL2 gene expression and neutrophil influx.

The purpose of the study reported here was to investigate the effects of in vitro exposure to solutions of AHB and AHS on expressions of selected cytokine genes in cultured equine primary BECs and to contrast these responses to those induced by endotoxin and hay dust to better understand the etiopathogenesis of IAD in horses. Given the potential for these cytokines to induce airway neutrophilia in experimental models of airway inflammation, we hypothesized that AHB would upregulate IL-8, IL-1β, and CXCL2 gene expressions in cultured equine BECs.

**Materials and Methods**

**Animals and sample collections**—Primary BEC cultures were established from lung samples obtained from 6 horses. The donor group consisted of 4 mares and 2 geldings (weight range, 450 to 550 kg) that had a median age of 15 years (range, 10 to 22 years). The group included 3 Quarter Horses, 1 Appaloosa, 1 Dutch Warmblood, and 1 Thoroughbred. Previously, all of the horses had been maintained on pasture for 2 to 3 months and were confirmed to be healthy on the basis of results of physical and endoscopic examinations. At the time of pasture turnout, horses had also been dewormed via administration of ivermectin and vaccinated against tetanus, influenza, eastern equine encephalomyelitis, western equine encephalomyelitis, and rabies. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cornell University and were in accordance with guidelines established by the National Institutes of Health.

Just prior to euthanasia, 500 mL of blood was aseptically collected from a jugular vein of each horse into a commercial blood collection bag and erythrocyte and nucleated cell concentrations were determined by use of a commercial cell counter. A 20-mL aliquot of blood was also obtained from each horse and allowed to coagulate at room temperature (approx 16°C) for 30 minutes. The serum was removed and centrifuged at 200 X g for 10 minutes to pellet any residual cells. The serum and whole blood samples were stored at 4°C until used. As a point of interest, erythrocyte and leukocyte numbers in 2 blood samples were determined immediately after collection and after 5 days of storage to evaluate the effect of short-term storage by analyzing total cell counts and cell distributions by use of a commercial cell counter.

Within 50 minutes after euthanasia, which was performed via IV injection of a solution of pentobarbital and phenytoin (95 mg/kg), the lungs of each horse were aseptically removed through a sterile thoracotomy that included resection of the ninth to 11th ribs. The left and right lung lobes were rinsed with sterile saline (0.9% NaCl) solution, placed in a sterile container, and immediately transported to the laboratory for processing in a laminar flow hood.

**Establishment of BEC cultures**—The BEC cultures were established as previously described. Briefly, bronchi (second- to 15th-generation airways) were removed from the lungs of each horse by use of sterile technique, transected into 3- to 5-cm-long segments, and washed 3 times with PBS solution containing 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 0.25 µg of amphotericin B/mL. Four airway segments were then placed in 50-mL tubes containing 30 mL of PBS solution with 0.1% protease 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.
The bronchial segments were transferred to a culture dish containing PBS solution with 10% fetal bovine serum, and the epithelial cells were gently detached by use of a scalpel blade. Cells were centrifuged at 200 × g for 10 minutes (4°C), washed twice, and resuspended (2 × 10⁵ cells/mL) in complete medium. One milliliter of the cell suspension was used to seed each 2-cm² well that previously had been coated with 500 µL of a coating solution (0.01% bovine serum albumin, 0.003% collagen, and 0.001% fibronectin in medium). and dried overnight (approx 12 hours). Cells were incubated at 37°C in humidified air with 5% CO₂, and culture medium was changed every 48 hours until 80% to 90% confluence was achieved on day 5. To confirm that confluent cells were ciliated epithelial cells, sample cultures from each horse were stained for cytokeratin, an epithelial cell marker, and evaluated microscopically as previously described.

Once cultures were confluent, the supernatant was removed from each cell culture well, the cells were gently washed, and the complete medium was replaced for 24 hours with medium devoid of hydrocortisone. Then, each well received 1 of 6 treatments as follows: 100 µL of PBS solution, 100 µL of HDS, 100 µL of LPS solution with a final concentration of 10 ng of LPS/mL in AHS (1% final concentration), 100 µL of AHS (1% final concentration in each well), 100 µL of whole blood (AHBl, 6.6 × 10⁶ RBCs), and 100 µL of whole blood diluted in PBS solution (AHB2, 1 × 10⁶ RBCs). Thus, there were 6 treatments evaluated in cells from each horse. The whole blood used in the 2 treatments also contained monocytes (mean count, 7.0 × 10³ µL), neutrophils (mean count, 2.2 × 10³ cells/100 µL), and lymphocytes (mean count, 2.6 × 10³ cells/100 µL).

Cells were incubated for 24 hours 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. The contents from each well were frozen at −80°C until gene expression analyses were conducted.

HDS preparation—The HDS was prepared as previously described. Briefly, flakes of dusty hay were agitated onto a clean surface and sieved through a grid (2 × 3 mm); fine particles were separated from coarse particles by use of a dual vortex vacuum cleaner. Ten milliliters of sterile saline solution was added to each gram of fine-particle hay dust, and the HDS was filtered through a mesh screen (100-μm pore size). The HDS was then exposed to gamma radiation (1.5 Mrad for 12 hours, with samples maintained on dry ice) to sterilize the solution and prevent bacterial and fungal overgrowth of the cell cultures. Particulates in the stock solution were counted by use of a hemocytometer, and endotoxin and β-glucan concentrations were measured by a commercial laboratory. Each milliliter of the stock HDS contained 10⁸ fungal particulates, 2 µg of endotoxin/mL, and 12.5 ng of β-glucan/mL. Thus, each well that was treated with the HDS contained 10⁶ fungal particulates, 2 ng of endotoxin, and 12.5 pg of β-glucan.

Measurement of gene expression—Total RNA was extracted separately from each cell culture well (each of 6 treatments for cells from each of the 6 horses) and the concentration and purity (spectrophotometric ratio, 260:280) were assessed. All samples had spectrophotometric ratios > 2. One microgram of RNA was treated with DNase to destroy any residual genomic DNA, and cDNA was synthesized. Gene expression was measured by use of a real-time reverse-transcriptase PCR assay. The target genes of interest were those for IL-8, CXCL2, and IL-1β, and each expression was normalized to the expression of a housekeeping gene, GAPDH. The GAPDH gene was selected because preliminary data had indicated that its expression in bronchial epithelium biopsy specimens was not altered by the presence of inflammation or by prior exposure to hay dust or LPS. The primer and probe sequences used in the kinetic PCR assay had been previously published. The PCR reaction mixtures had a final volume of 2.5 µL (2.5 µL of cDNA and 25 µL of the master mix). For each cDNA sample, triplicate reactions were performed on each plate for detection of the target genes. Negative controls (lacking cDNA) and positive controls (cDNA from LPS-stimulated monocytes) were included on each plate.

The endpoint used in the real-time reverse transcriptase PCR quantifications was the Ct at which the amplicon was detected. The Ct ranges from 0 to 40. Gene expression was reported as the ΔCt or as the fold change in the target gene. 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 Ct value, the more cDNA (ie, mRNA) was contained in a sample. For the second method, the fold change was calculated as 2−ΔΔCt, where ΔΔCt represents the difference between the ΔCt of specific treatments (eg, LPS solution-treated cells, compared with PBS solution-treated cells).

Statistical analysis—Treatment effects (6 treatments of samples from 6 horses) on the ΔCt were analyzed by use of Kruskal-Wallis tests. Post hoc comparisons were performed by use of Wilcoxon signed rank tests. Because of the multiple comparisons that were made, a Bonferroni correction was applied and was calculated by dividing α (0.05) by the number of target genes (3) compared (resultant = 0.017). All computations were performed by use of a statistical software program.

Results

As a point of minor interest, the erythrocyte and leukocyte numbers in 2 blood samples in the present study were assessed immediately after collection and after 5 days of storage. Although erythrocyte numbers did not change with storage, total WBC counts had decreased approximately 20% from the initial collection values (data not shown) because of a reduction in granulocyte and monocyte numbers.

Analyses revealed that there was no significant effect of treatment on GAPDH expression in the BEC cultures. For the 6 treatments, the mean ± SD Ct values were 18.3 ± 0.7 for PBS solution, 18.2 ± 0.7 for AHS, 18.7 ± 0.4 for AHB1, 18.7 ± 0.4 for AHB2, 18.2 ± 0.7 for HDS, and 18.7 ± 0.4 for LPS solution.

Box-and-whisker plots of ΔCt values for IL-8, CXCL2, and IL-1β gene expressions in cultured BECs...
following each of the 6 treatments were created (Figure 1). Relative to the PBS solution, all treatments with the exception of AHS resulted in upregulation of the expression of the 3 target genes ($P < 0.017$). The fold increases in each target gene expression following the 5 treatments relative to the target gene expression in PBS solution were summarized (Table 1). Treatment of cultured BECs with AHB was associated with a dose-dependent upregulation of target gene expression; AHB1 exposure resulted in a 20-fold increase in gene expression of IL-8, a 6-fold increase in gene expression of CXCL2, and a 260-fold increase in gene expression of IL-1$\beta$, relative to findings following PBS solution challenge. Treatment with AHB2 (the erythrocyte concentration of which was 15% of that of AHB1) resulted in upregulation of the gene expressions of IL-8, CXCL2, and IL-1$\beta$ that were 44%, 52%, and 18%, respectively, of that induced with AHB1 treatment. Treatment of cultured BECs with HDS or LPS solution also resulted in approximately 20-fold increases in IL-8 and IL-1$\beta$ gene expressions, compared with findings following PBS solution exposure. In contrast, only 8- and 7-fold increases in CXCL2 gene expression occurred following exposures to HDS and LPS solution, respectively. The fold increases in IL-8 and CXCL2 gene expression associated with HDS or LPS solution treatment were not different ($P > 0.017$) from changes induced following AHB1 treatment. In contrast, the fold increase in IL-1$\beta$ gene expression following treatments with HDS ($P = 0.016$) or LPS solution ($P = 0.016$) was significantly less than that following AHB1 treatment.

### Discussion

To our knowledge, the present study is one of the first to demonstrate that AHB but not AHS induces a dose-dependent increase in the gene expression of pro-inflammatory cytokines and neutrophil chemoattractants in cultured equine BECs. Indeed, the IL-8 and CXCL2 gene expression responses of the BECs to AHB treatments were similar to those induced with HDS or a solution of LPS, one of the components of hay dust. Even more noteworthy was the finding that the upregulation of IL-1$\beta$ gene expression was greater following AHB treatments than it was following HDS or LPS solution exposure, a response that could further augment chemokine expression in the cultured BECs. These
data provided supportive evidence that following exposure to pulmonary hemorrhage in vivo, either as a consequence of strenuous exercise, trauma, or severe microbial infections, the airway epithelium contributes to the inflammatory cell influx.

Previous investigators have reported that instillation of AHB into the airways of horses induces an inflammatory reaction as evidenced by alterations in respiratory mechanics or increases in the total nucleated cell and neutrophil counts in BAL fluid samples.16,17 The specific pulmonary cells that respond to the blood, secrete chemokines, and induce neutrophil influx into the airways as well as their importance in the development of the inflammatory process have not been ascertained. Based on the observation that the percentage of alveolar macrophages containing either erythrocytes (erythropages) or hemosiderin (hemosiderophages) increases within 24 hours after AHB instillation,15,20 it has been logical to assume that alveolar macrophages orchestrate the inflammatory reaction by the release of chemokines and reactive oxygen species.20 However, the kinetics of their cytokine responses relative to that of other cells (eg, airway epithelial cells) remains to be established.

One potential limitation of the present study was that it focused on the responses of cultured equine BECs to AHB exposure without identifying the mechanism of chemokine upregulation. Specifically, the study design did not permit us to determine what cellular or molecular components in the blood (leukocytes, erythrocytes, free hemoglobin, or proinflammatory cytokines) mediated the epithelial responses and whether there was any cross-communication between the epithelial cells and WBCs contained within the AHB. For example, when human BECs are cocultured with human peripheral blood monocytes for 24 hours, the latter assume a phenotype closely resembling that of an activated mature alveolar macrophage.31 When cocultures of human alveolar macrophages and human BECs are exposed to particulates, there is a synergistic upregulation of tumor necrosis factor-α, IL-1β, IL-6, and IL-8 mRNAs and protein synthesis derived from both cell types.32

Other researchers have shown that peripheral blood neutrophils interact with BECs; Jagels et al.33 found that neutrophil adherence to human BECs is facilitated by IL-8, complement 5a, and intercellular adhesion molecule-1, the epithelial expression of the latter being upregulated by IL-1, tumor necrosis factor-α, and IL-8.33 The outcome of neutrophil-epithelial cell adhesion is an enhancement of neutrophil function via cytokine production, an increase in the permeability of the epithelial cells (which facilitates leukocyte emigration), or an induction of epithelial cell apoptosis.34-36 All of these actions have the potential to cause persistence of pulmonary inflammation in vivo.

Peripheral blood lymphocytes also adhere to and interact with the airway epithelium through chemokine receptors and through several adhesion molecules, such as αEβ7 integrin and lymphocyte endothelial-epithelial cell adhesion molecule.37,38 As a result of these interactions, chemokine secretion by BECs is enhanced and facilitates retention of lymphocytes within the airways. The net result is to bolster immune responses against microbial infections or to enhance tumor cell surveillance.

Thus, to ascertain whether any of these modulating effects of leukocytes on the cultured BECs occurred in the present study, we would have had to also perform leukoreduction of the blood39,40 prior to treating the BEC cultures. As a point of interest, we determined erythrocyte and leukocyte numbers in 2 blood samples immediately after collection and after 5 days of storage in the present study. Although erythrocyte numbers did not change with storage, total WBC counts had decreased approximately 20% from the initial collection values because of reductions in granulocyte and monocyte numbers. It is possible that we underestimated the magnitude of the chemokine responses in the epithelial cells by using 5-day-old blood that had fewer leukocytes than did fresh samples.

Finally, the modulating effects of erythrocytes or extruded hemoglobin on epithelial cell chemokine gene expression should be considered. Unfortunately, there is a paucity of studies that have sought to investigate any deleterious or modulating effects that erythrocytes have on the pulmonary epithelium ex vivo. Although free hemoglobin has been shown to induce an oxidant stress in various tissues,41 including the vascular endothelium, its effects on the bronchial epithelium have not been reported, to our knowledge.

In the present study, exposure of cultured equine BECs to AHB induced a significant increase in the gene expressions of 3 important neutrophil chemokines. Overall, these chemokine upregulations in vivo may contribute to the development of pulmonary inflammation following hemorrhage in strenuously exercising horses.
References


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