Phagocytosis, bacterial killing, and cytokine activation of circulating blood neutrophils in horses with severe equine asthma and control horses

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OBJECTIVE
To evaluate in vitro phagocytosis and bactericidal activity of circulating blood neutrophils in horses with severe equine asthma and control horses and to determine whether circulating blood neutrophils in horses with severe equine asthma have an increase in expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α and the chemokine interleukin (IL)-8 and a decrease in expression of the anti-inflammatory cytokine IL-10 in response to bacteria.

ANIMALS
6 horses with severe equine asthma and 6 control horses.

PROCEDURES
Circulating blood neutrophils were isolated from horses with severe equine asthma and control horses. Phagocytosis was evaluated by use of flow cytometry. Bactericidal activity of circulating blood neutrophils was assessed by use of Streptococcus equi and Streptococcus zooepidemicus as targets, whereas the cytokine mRNA response was assessed by use of a quantitative PCR assay.

RESULTS
Circulating blood neutrophils from horses with severe equine asthma had significantly lower bactericidal activity toward S. zooepidemicus but not toward S. equi, compared with results for control horses. Phagocytosis and mRNA expression of TNF-α, IL-8, and IL-10 were not different between groups.

CONCLUSIONS AND CLINICAL RELEVANCE
Impairment of bactericidal activity of circulating blood neutrophils in horses with severe equine asthma could contribute to an increased susceptibility to infections. (Am J Vet Res 2018;79:455–464)

Severe equine asthma (also known as recurrent airway obstruction or heaves) is a common chronic pulmonary inflammatory disease affecting mature horses in the Northern Hemisphere. Affected horses develop labored breathing at rest that is associated with bronchoconstriction, mucus secretion, and airway remodeling when exposed to aeroallergens during stabling and hay feeding. Infiltination of neutrophils within the airways is a characteristic finding of severe equine asthma and occurs within hours after exposure to moldy hay. Although the exact role neutrophils play in the pathogenesis of the condition is not clear, there is evidence that circulating blood neutrophils are activated in horses with severe equine asthma.

Neutrophils are the primary cell type involved in the initial host defense against pathogenic microorganisms and play an important role in clearance of pathogenic microorganisms through engulfment and degradation. Bacterial growth on microbial culture of tracheal secretions and samples from horses with bronchiectasis, which possibly developed as a consequence of chronic bacterial colonization of the airways, has been reported secondary to severe equine asthma. Humans with bronchiectasis and asthma have a reduced ability to kill Candida spp and Staphylococcus aureus, respectively. Collectively, these findings suggest that the bactericidal capacity of neutrophils in equine chronic respiratory diseases, such as severe equine asthma, could be altered. This could potentially contribute to the development of secondary bacterial infection in severely asthmatic horses.

Several in vitro assays exist for the evaluation of bactericidal activity and phagocytic capacity of neutrophils. For horses, bactericidal activity has been assessed by use of chemiluminescence assays, nitroblue tetrazolium dye reduction tests, and incubation of neutrophils and bacteria with subsequent counting of viable bacteria after incubation. Pha-
gocytosis assays typically assess the ability of a cell to attach to or to internalize particles. Phagocytosis in horses has been evaluated by use of light microscopy and flow cytometry. Flow cytometry analysis is a rapid and reproducible method for analyzing phagocytosis in large numbers of cells on a cell-by-cell basis.

Equine neutrophils are capable of expressing an array of proinflammatory and chemotactatant cytokines, many of which are upregulated during severe equine asthma. Currently it is recognized that these neutrophils are capable of modulating their cytokine response on the basis of the antigens they encounter. For instance, when stimulated with IL-4, a cytokine upregulated in the lungs of horses with severe equine asthma, expression of IL-1β by circulating blood neutrophils is lower, compared with expression after activation by bacterial extracts. Therefore, the complex inflammatory milieu in the lung tissues of horses with severe equine asthma could differentially activate neutrophils and influence their cytokine response to bacterial infection.

On the basis of these observations, we hypothesized that phagocytosis or bacterial killing (or both) by circulating blood neutrophils in horses with severe equine asthma would be less than in healthy horses, and that circulating blood neutrophils would have altered expression of inflammatory mediators in response to bacteria. Thus, the first objective of the study reported here was to compare phagocytosis and bacterial killing of Streptococcus equi and Streptococcus zooepidemicus by circulating blood neutrophils of horses with clinical signs of severe equine asthma and control horses. These bacteria were selected because S. equi and S. zooepidemicus, which are β-hemolytic and Lancefield group C streptococci, are commonly involved in equine diseases. Streptococcus zooepidemicus is a part of the normal flora in horses and the organism most frequently cultured (72.0%) from infections in which β-hemolytic streptococci are isolated. Streptococcus equi causes strangles, a contagious, acute, and supplicative lymphadenitis in horses. The second objective of the study reported here was to determine whether circulating blood neutrophils of horses with severe equine asthma have an increase in the expression of the proinflammatory cytokine TNF-α and the chemokine IL-8 and a decrease in expression of an anti-inflammatory cytokine IL-10, compared with expression in horses without respiratory disease.

Materials and Methods

Animals

Twelve adult mixed-breed horses (10 mares and 2 geldings) from a university research herd were included in the study. Six horses with severe equine asthma had a history of chronic respiratory tract disease and abnormal lung function following exposure to moldy hay. Six control horses had no history or clinical signs of respiratory tract disease and had a maximum transpulmonary pressure of < 10 cm H2O following exposure to moldy hay. Horses were kept in pastures with no additional food supplementation for 3 months prior to the study. Horses had not been medicated for at least 3 months prior to the study, and they were regularly vaccinated and dewormed.

The 2 groups of horses were stabled in the same barn during the experimental period. Horses were fed dry timothy hay and sweet feed twice each day; straw bedding was used for all horses. All stabled horses were exposed to moldy hay. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Montreal.

Clinical score and pulmonary function

After the horses were exposed to moldy hay, a clinical score was assessed by 2 observers (JMV and M-PL) as described elsewhere. Briefly, a score from 0 to 4 was assigned for abdominal movement (0 = no abdominal movement and 4 = severe and marked abdominal movement) and nasal flaring (0 = no flaring and 4 = severe and continuous flaring). Abdominal and nasal flaring scores were added (maximum score, 8). Scores ≥ 5 indicated airway dysfunction.

Measurements of respiratory mechanics were performed as described elsewhere. Briefly, flow rate was measured by use of a heated pneumotachograph and associated differential pressure transducer fitted into a mask that was placed over the nose of each horse. Esophageal pressure was measured with a balloon sealed onto the end of a polyethylene catheter placed in the distal third of the esophagus. Transpulmonary pressure was determined by use of a differential pressure transducer, and the esophageal pressure was subtracted from the mask pressure. Signals of the transducers were amplified and analyzed by use of a digital-analog converter and computer equipped with data acquisition and analysis software. Signals were sampled at a frequency of 120 Hz for 90 seconds, and all valid breaths were used for analysis. Values of pulmonary resistance and elastance were obtained for each breath by applying a multiple regression equation for a single compartment model of the lungs.

BAL

One week after horses were exposed to moldy hay, BAL was performed. Horses were sedated and maintained in a standing position. A flexible endoscope and 2 boluses (250 mL/bolus) of sterile isotonic saline (0.9% NaCl) solution were used, as described elsewhere. Total nucleated cells were counted with a hemacytometer. Cytocentrifugation slides were stained with a modified Wright stain, and a differential count was performed on 400 cells.

Isolation of circulating blood neutrophils

At the same time as the BAL, a blood sample (10 mL) was collected via jugular venipuncture into
sterile EDTA-containing evacuated tubes. Blood samples were stored at room temperature (20°C) until processed; all blood samples were processed within 30 minutes after collection. Circulating blood neutrophils were isolated by use of density gradient centrifugation performed in accordance with the manufacturer’s instructions. Purified circulating blood neutrophils were then suspended in RPMI 1640 medium and supplemented with normal pooled equine serum (60% RPMI 1640 and 40% normal equine serum; final concentration, 5 × 10⁶ cells/mL). Total cell counts were determined with a hemacytometer, and viability was assessed by use of the trypan blue dye exclusion test.

**Bacterial strains and growth conditions**

Two strains of pathogenic *Streptococcus* spp were used: *S. equi* and *S. zooepidemicus*. In addition, a nonencapsulated strain of *Streptococcus suis* (strain B218) was used as a positive control sample. *Streptococcus suis* B218 is a well-characterized strain that is highly susceptible to phagocytosis, and it is considered an appropriate control sample to validate a phagocytosis assay. *Bacteria* were grown overnight on sheep blood agar plates at 37°C. Isolated colonies were used as inoculum for 10 mL of Todd-Hewitt broth, which was incubated for 16 hours at 37°C with agitation. Optimal working cultures were obtained by inoculating 0.2 mL of the *S. zooepidemicus* overnight suspension with 9.8 mL of RPMI 1640 medium and 0.5 mL of the *S. equi* overnight suspension with 9.5 mL of RPMI 1640 medium; cultures were incubated for 2 hours at 37°C with agitation. *Streptococcus suis* B218 was grown as described elsewhere. Logarithmic-phase bacteria were washed 3 times in PBS solution and then diluted to a concentration of 5 × 10⁶ CFUs/mL in RPMI 1640 medium. The number of CFUs per milliliter in the final suspension was determined by plating samples onto Todd-Hewitt agar.

**Bactericidal assay**

An aliquot (25 mL) containing circulating blood neutrophils at a concentration of 5 × 10⁶ cells/mL diluted in RPMI 1640 medium and normal equine serum (60% RPMI 1640 medium and 40% normal equine serum) was mixed in microtubes with 0.25 mL of *S. suis* B218 at 2 × 10⁴ CFUs/mL (positive control sample) or with 0.25 mL of *S. equi* or *S. zooepidemicus* at 2 × 10⁷ CFUs/mL. These microtubes were then incubated at 37°C and 5% CO₂ for 90 minutes (*S. suis* B218) or 60 minutes (*S. equi* and *S. zooepidemicus*). Preliminary experiments on the kinetics for killing of *S. suis* B218, *S. equi*, and *S. zooepidemicus* by neutrophils revealed that killing was optimal for these experimental conditions (data not shown). After incubation with circulating blood neutrophils was completed, cells were lysed with sterile water, and viable bacterial counts were performed on Todd-Hewitt agar plates with an automated cell counter. Tubes with bacteria alone were treated similarly and used as control samples. Results were expressed as the percentage of bacteria that were killed.

**Phagocytosis determined by use of flow cytometry**

Red fluorescent polystyrene beads (1 μm in diameter; 10¹⁰ beads/mL) were washed in PBS solution and resuspended in RPMI 1640 medium at a concentration of 2.5 × 10⁸ beads/mL. An aliquot (0.2 mL) containing circulating blood neutrophils at a concentration of 5 × 10⁶ cells/mL was mixed in microtubes with 0.2 mL of fluorescent beads; microtubes then were incubated at 37°C and 5% CO₂ for 30 minutes. Extracellular beads were removed through 3 washes with PBS solution, and cells were resuspended in 0.4 mL of sorting buffer (PBS solution–1% fetal bovine serum). A solution of 0.2 mL of circulating blood neutrophils at a concentration of 5 × 10⁶ cells/mL without fluorescent beads was used as a negative control sample. All incubations were performed in the dark. Flow cytometry was performed with a flow cytometer. Data for 30,000 events were acquired and analyzed by use of software. The percentage of circulating blood neutrophils that had ingested beads and thereby held red fluorescence was calculated.

**Confocal microscopy**

Neutrophils were incubated with fluorescent beads as described previously. Extracellular beads were removed through 3 washes with PBS solution, and cells were stained by incubation with an antibody against the surface neutrophil marker CD90 at 4°C. Cells were washed 3 times in PBS solution and incubated for 5 minutes with phycoerythrin-coupled goat anti-mouse IgM antibody (diluted 1:500 in PBS solution). Cells were washed twice, examined with a confocal microscope integrated into an enhanced imaging system, and analyzed by use of imaging software.

**Neutrophil stimulation assays**

An aliquot (0.5 mL) of circulating blood neutrophils at a concentration of 2 × 10⁶ cells/mL, diluted in RPMI 1640 medium supplemented with equine serum (60% RPMI 1640 medium and 40% equine serum), was mixed in microtubes with 0.5 mL of *S. equi* or *S. zooepidemicus* at a concentration of 10⁴ CFUs/mL and incubated for 4, 8, and 12 hours in an incubator at 37°C and 5% CO₂. The RPMI 1640 medium and LPS from *Escherichia coli* O111:B4 (2 μg/mL) were used as negative and positive control samples, respectively. Gene expression for TNF-α was evaluated at 4, 8, and 12 hours, and gene expression for IL-10 and IL-8 was evaluated at 8 and 12 hours. At each time point, neutrophils were centrifuged, and the pellet was resuspended in 1 mL of reagent and frozen at -70°C for subsequent analysis.

**RNA extraction and reverse transcription**

Total RNA extraction from the reagent was performed in the presence of 10 μg of glycogen in accordance with the manufacturer’s instructions. The RNA pellets were air dried and suspended in RNase-
DNase-free water. The concentration and purity of RNA was evaluated with a spectrophotometer. Two hundred nanograms of total RNA was reverse transcribed in the presence of 60 U of ribonuclease inhibitor by use of 500 U of reverse transcriptase in accordance with the manufacturer’s instructions, except that the reaction was performed in a final volume of 30 µL. Random hexamers (225 ng) were used in addition to 500 ng of oligo(dT)12-18. The cDNA samples were stored at -20°C.

Real-time quantitative PCR assay

The cDNA was amplified by use of a PCR amplification kit with a real-time PCR assay detection system. All PCR assay amplifications included an enzyme activation step of 3 minutes at 98°C, which was followed by 40 cycles of a denaturing step for 3 seconds at 98°C and an annealing-extension step for 5 seconds at 57°C. The final reaction volume was 10 µL that contained 250nM each of forward and reverse primers used to amplify the target genes (TNF-α, IL-8, IL-10, and ubiquitin; Appendix). Primer sequences for GAPDH have been published in another study. Ubiquitin and GAPDH were selected by use of the PCR assay detection system as the most stable genes among 4 candidates (which also included β-actin and 18S) and were used for normalization. Amplification efficiencies ranged between 94% and 109%. Differences between samples were calculated by use of the 2-ΔΔCt method. Gene expression data at each time point for each horse were expressed as the fold difference over that of results for the nonstimulated neutrophils (ie, negative control sample, which was assigned a value of 1). This quantification method helped eliminate run-to-run variations because all samples for a given time point and each horse were assayed on the same PCR assay plate. Differences in baseline gene expression (unstimulated circulating blood neutrophils in RPMI 1640 medium) were not reported.

Statistical analysis

Bactericidal and phagocytic activities of neutrophils from horses with severe equine asthma and control horses were compared by use of Student t tests. The PCR assay data were logarithmically (log2) transformed to normalize distribution and included in a repeated-measures linear model; group (horses with severe equine asthma vs control horses) was used as a between-subject factor, whereas time (4, 8, and 12 hours) and stimulation (S zooepidemicus, S equi, and LPS) were used as within-subject factors. Differences for within-subject and between-subject factors were further evaluated by use of a priori contrasts with sequential Bonferroni correction for multiple testing. Statistical software was used for the analysis. Results were expressed as mean ± SD or mean ± SEM. Differences between groups were considered significant at P ≤ 0.05. Other differences were considered significant at P < 0.01.

Results

Animals

The 6 horses with severe equine asthma (5 mares and 1 gelding) had a mean ± SD body weight of 487.8 ± 38.8 kg, whereas the control horses (5 mares and 1 gelding) had a mean body weight of 486.2 ± 14.3 kg. Horses with severe equine asthma were significantly (P = 0.02) older (21.0 ± 3.0 years) than control horses (15.6 ± 3.4 years).

Clinical evaluation of horses

Horses with severe equine asthma had clinical exacerbation of the disease as indicated by high values for pressure decrease per unit length, pulmonary resistance, and elastance, whereas lung function of the control horses remained within reference limits (Figure 1). Mean ± SD percentage of neutrophils in BAL fluid was significantly (P < 0.001) higher in horses with severe equine asthma (37.2 ± 3.7%) than in control horses (12.3 ± 6.1%).

Isolation of circulating blood neutrophils

Purity of the neutrophils isolated from blood samples was > 99%. Viability of the isolated neutrophils was > 98%.

Bactericidal activity of circulating blood neutrophils against S equi and S zooepidemicus

Circulating blood neutrophils in severely asthmatic horses had significantly (P = 0.046) lower bactericidal activity (mean ± SD, 8.5 ± 10.6%) against S zooepidemicus than did control horses (36.5 ± 25.8%; Figure 2). Bactericidal activity against S equi for neutrophils from horses with severe equine asthma (7.3 ± 11.8%) did not differ significantly (P = 0.10) from that of neutrophils from control horses (23.7 ± 17.8%). Bactericidal activity of neutrophils against S suis (positive control sample) did not differ significantly (P = 0.68) between groups of horses (horses with severe equine asthma, 95.6 ± 3.9%; control horses, 94.3 ± 6.6%).

Phagocytosis capacity of circulating blood neutrophils

No significant (P = 0.91) differences were observed in the phagocytic activity of circulating blood neutrophils from severely asthmatic horses and healthy control horses (Figure 3). In addition, the number of phagocytosed fluorescent beads per neutrophil, as determined by use of confocal microscopy, was similar between groups (Figure 4).

Gene expression induced in circulating blood neutrophils by S equi and S zooepidemicus

Expression of TNF-α and IL-8 by circulating blood neutrophils was significantly (P < 0.001) upregulated by both bacteria. However, there was no effect of the
Expression of IL-8 was significantly ($P < 0.001$) higher for *S. zooepidemicus* stimulation of circulating blood neutrophils than for *S. equi* or LPS stimulation of circulating blood neutrophils after 8 hours, whereas both *S. zooepidemicus* and *S. equi* induced significantly ($P = 0.002$) higher IL-8 expression than did LPS after 12 hours. In contrast, all stimuli had a similar effect on IL-10 expression (Figure 5).

**Discussion**

Circulating blood neutrophils of horses with severe equine asthma are activated, compared with results for those of healthy horses. However, it is unknown whether the disease affects the phagocytic or bactericidal activity (or both) of circulating...
blood neutrophils. Results of the study reported here indicated that the bactericidal activity of circulating blood neutrophils against *S. zooepidemicus*, but not against *S. equi*, was lower in severely asthmatic horses. This difference between the 2 bacteria can be explained by an inability of neutrophils to internalize or destroy *S. equi*. Partial failure of neutrophils to phagocytose and kill *S. equi* appears to be attributable to several morphological characteristics of *S. equi*, including a combination of the hyaluronic acid capsule, anti-phagocytic scanning electron microscopy protein, M-protein, and other undetermined anti-phagocytic factors released by the bacteria. Virulent isolates of *S. equi* from horses with strangles typically are highly encapsulated, whereas nonencapsulated mutants are much less virulent. The antiphagocytic capsule greatly reduces the number of streptococci that become associated with the surface of neutrophils and that subsequently are ingested and killed.

A decrease of killing activity against *Candida* spp by polymorphonuclear neutrophilic leukocytes was observed in humans with asthma and was believed to contribute to an increased susceptibility to infection in these patients. Circulating blood neutrophils of humans with bronchiectasis also reportedly have a reduced ability to kill *S. aureus*, compared with the ability for those of healthy control subjects. Results of the present study suggested that the reduced killing capacity of circulating blood neutrophils could promote bacterial colonization and secondary infections in horses with severe equine asthma. Although overwhelming bacterial bronchopneumonia is uncommon in horses with severe equine asthma, there are anecdotal reports of pulmonary infectious diseases in severely asthmatic horses. Investigators of 1 study reported that pulmonary chlamydial infections with inflammation were more...
frequent in horses with severe equine asthma than in control horses. In another study, investigators described 3 severely asthmatic horses with bronchiectasis. This irreversible destruction of the airway architecture leads to localized or diffuse chronic dilatation, impaired mucociliary clearance, bacterial colonization, and recurrent infection. In addition, a strong association between isolation of *S. zooepidemicus*, *Streptococcus pneumoniae*, *Actinobacillus* spp, *Pasteurella* spp, and *Mycoplasma equi* from tracheal wash solution and the presence of inflammatory airway disease has been reported. However, whether these bacteria play an important role in the pathogenesis of inflammatory airway disease has not yet been determined.

In the study reported here, the impaired bactericidal capacity detected in horses with severe equine asthma was likely unrelated to a reduced number of internalized bacteria because there was no difference in the phagocytic activity between neutrophils from severely asthmatic and control horses as determined by use of flow cytometry and confocal microscopy. These results support the findings that the phagocytic activity against *S. aureus* or yeast cells is unaffected in horses with severe equine asthma. A similar discrepancy between neutrophil function and phagocytosis was reported in humans with asthma and bronchial bacterial infection, whereby random migration, nitroblue tetrazolium dye reduction, and *Candida* spp killing activity were markedly decreased in polymorphonuclear neutrophilic leukocytes; however, phagocytosis was unaffected in both groups of patients in that report. Phagocytosis is triggered by the binding of opsonized bacteria through opsonin or nonspecific glycosylated receptors that recognize certain lectins on target bacteria. Two bactericidal processes are activated concomitantly with phagocytosis: the oxidative burst (production of cytotoxic reactive oxygen species and reactive nitrogen species) and the degranulation and release of contents of azurophilic and specific granules into the phagosome. Extracellular bactericidal activities have also been described for neutrophils. Intracellular or extra-

**Figure 5** — Gene expression of TNF-α (A through C), IL-8 (D through F), and IL-10 (G through I) induced in circulating blood neutrophils obtained from 6 control horses (white circles) and 6 horses with severe equine asthma (black circles) and stimulated by incubation with *S. zooepidemicus* (10⁴ CFUs/mL; A, D, and G), *S. equi* (10⁴ CFUs/mL; B, E, and H), or LPS (2 µg/mL; C, F, and I) for up to 12 hours. Results were obtained by use of a real-time quantitative PCR assay and represent the fold increase (as calculated by use of the 2^−ΔΔCt method) over that of unstimulated neutrophils (ie, negative control sample, which was assigned a value of 1 for each horse at each time point). Ubiquitin and GAPDH were used as reference genes to normalize gene expression. Notice that the scale on the y-axis for IL-8 (panels D through F) differs from the scale on the y-axis for TNF-α and IL-10. *Value differs significantly (P < 0.01) from the value for LPS stimulation at the same time point. †Value differs significantly different (P < 0.01) from the value for *S. equi* stimulation at the same time point.
cellular (or both) bactericidal mechanisms affected by severe equine asthma warrant further investigation. Moreover, modulation of the innate immune response is another important component of neutrophil function.51,52

Horses with severe equine asthma were significantly older than control horses of the present study. It is highly unlikely that this difference impacted results of this study because age-related changes of the immune system primarily impact adaptive immunity. In healthy aged horses, quantity of circulating neutrophils,53 ability of neutrophils to adhere, oxidative burst, and phagocytosis are all unchanged,54 compared with results for younger adult horses. An age-related increase in gene expression of proinflammatory cytokines produced by peripheral blood mononuclear cells has been reported, but it is unclear how an age-associated imbalance in cytokines may influence neutrophil activity.

Pulmonary neutrophilia (> 5%) was also evident for the BAL samples of control horses of the present study. This finding is not unusual for healthy horses and may be the result of stabilizing in a dusty environment.55,56 However, this effect is less common in healthy horses than in horses with severe equine asthma and usually resolves despite continuous exposure to stable dust. Different inflammatory pathways likely contribute to the recruitment of inflammatory cells in the 2 groups because healthy horses with airway neutrophilia do not develop altered lung function after antigenic exposure,41 which is in contrast to the effects in horses with severe equine asthma.

In the present study, blood samples were collected 1 week after horses were exposed to moldy hay. This allowed a sufficient interval for horses with severe equine asthma to develop clinical signs and stabilize airway obstruction. The timing selected for blood collection should be taken into consideration when evaluating neutrophil expression data. Investigators of 1 study4 detected priming of equine circulating blood neutrophils in addition to transient neutrophilic leukocytosis 24 hours after challenge exposure to hay or straw, with results returning to preexposure values within 4 days after cessation of exposure. For the study reported here, we assumed that neutrophil cytokine expression 1 week after antigen exposure would be sufficiently stable to allow evaluation.

Ex vivo production of proinflammatory (IL-8 and TNF-α) and anti-inflammatory (IL-10) cytokines by circulating blood neutrophils in response to various Streptococcus spp was compared between control horses and severely asthmatic horses. Equine neutrophils are capable of expressing an array of proinflammatory and chemotactic cytokines. In the present study, we selected a representative inflammatory cytokine involved in bacterial sepsis and inflammatory diseases (TNF-α) and a potent chemokine for neutrophils (IL-8, which is also produced by these cells). Finally, because equine asthma may induce modulation of neutrophil function, a regulatory cytokine (IL-10) was also evaluated. As determined by use of the assay conditions for the present study, ex vivo mRNA expression of these cytokines by circulating blood neutrophils was not significantly increased in horses with clinical exacerbation of severe equine asthma. Similarly, no significant differences in the concentrations of proinflammatory cytokines (TNF-α) and chemokines (IL-8) produced by circulating blood neutrophils in horses with severe equine asthma and control horses were detected after 5 hours of an antigenic challenge in another study.29 Also, the serum IL-10 concentration is not significantly different between control and horses with severe equine asthma at 7 and 10 days after antigenic challenge.57 In another study,50 investigators reported ex vivo overexpression of TNF-α for circulating blood neutrophils (which were obtained from severely asthmatic horses that did not have clinical signs of the disease) stimulated by microbial products (LPS) for 5 hours. A possible explanation for this difference could be that in severely asthmatic horses without clinical signs of the disease, circulating blood neutrophils are already maximally upregulated by exposure to microbial products (bacteria). Considering that there is variable expression of cytokines and chemokines in the first hours of antigen or LPS-bacterial stimulation in vivo and ex vivo, it is difficult to determine the role of these cytokines and chemokines in the early phases of recruitment and activation of inflammatory cells involved in the pathogenesis of severe equine asthma.

For the study reported here, the bactericidal capacities of circulating neutrophils against S. zooepidemicus were lower in horses with severe equine asthma than in control horses, although phagocytosis and mRNA expression of TNF-α, IL-8, or IL-10 were unaltered. Reduced bactericidal capacities of circulating blood neutrophils against S. zooepidemicus could potentially contribute to the development of secondary infections in severely asthmatic horses.

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Footnotes
a. RHT-Infodat, RHT-INFODAT INC, Brossard, QC, Canada.
b. Vacutainer, Becton Dickinson, Mississauga, ON, Canada.
c. Lympholyte-poly, Cedarlane Laboratories, Burlington, ON, Canada.
d. Gibco, Ottawa, ON, Canada.
e. Vacutainer, Becton Dickinson, Mississauga, ON, Canada.
g. Fluosphere, Invitrogen, Eugene, Ore.
h. FACSCalibur, BD Biosciences, San Jose, Calif.
i. CellQuest Pro, BD Biosciences, San Jose, Calif.
References

37. Lavoie JP, Magnhi K, Desnoyers M, et al. Neutrophilic airway inflammation in horses with heaves is characterized


**Appendix**

Primers used in a real-time PCR assay for the quantification of cytokines expressed by equine neutrophils.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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