Influx of neutrophils and persistence of cytokine expression in airways of horses after performing exercise while breathing cold air

Michael S. Davis, DVM, PhD; Caroline C. Williams, BS; James H. Meinkoth, DVM, PhD; Jerry R. Malayer, PhD; Christopher M. Royer, BS; Katherine K. Williamson, DVM; Erica C. McKenzie, BVSc, PhD

Objective—To determine effects of exercise performed while breathing cold air on expression of cytokines and influx of neutrophils in airways of horses.

Animals—9 adult horses.

Procedures—In a crossover study, bronchoalveolar lavage fluid (BALF) was obtained 24 and 48 hours after each of 2 submaximal exercise sessions performed by horses while breathing warm (25°C) or cold (–5°C) air. Total and differential nucleated cell counts were determined for each BALF sample. Relative mRNA expression of cytokines in BALF cells was quantified by use of a reverse transcription–PCR assay.

Results—Horses had a modest but significant influx of neutrophils into the airways 24 hours after a single exercise session while breathing cold air. No other cell types were increased at 24 or 48 hours after exercising while breathing cold air. Continued increases in expression of cytokines interleukin (IL)-5 and -10 as well as proinflammatory cytokines IL-1, -6, and -8 were detected 24 hours after exercising while breathing cold air. Forty-eight hours after exercising while breathing cold air, expression of IL-10 was still higher than that for IL-10 after horses exercised while breathing warm air. Expression of tumor necrosis factor-α was significantly increased at 48 hours after exercising while breathing cold air.

Conclusions and Clinical Relevance—Exposure of intrapulmonary airways to cold air alters immunologic responses of horses for at least 48 hours. The increased expression of cytokines that suppress cell-mediated immunity may predispose athletes to viral infections of the respiratory tract following exercise in cold weather. (Am J Vet Res 2007;68:185–189)

Human athletes who routinely perform strenuous exercise in cold conditions have a high prevalence of chronic airway inflammation and hyperreactivity, which is commonly referred to as ski asthma because of its original description in cross-country skiers. This suggests that such activity may be capable of de novo induction of an asthmalike syndrome. In addition, human athletes who perform strenuous exercise are predisposed to postexercise viral infections, particularly of the respiratory tract. This predisposition, commonly referred to as the open window period, has been widely reported in numerous populations of human athletes, but the cause of the overall predisposition, or the reason for the apparent selectivity for the respiratory tract, is unknown.

In another study conducted by our laboratory group, we experimentally created a condition in horses that is analogous to ski asthma in humans and have used such horses to evaluate the effects of strenuous exercise while breathing cold air on pulmonary physiologic processes and determine whether exercise while breathing cold air can cause chronic airway disease. Evaluation of those horses confirmed failure of the nasal passages to fully warm inspired air, which led to exposure of the trachea and bronchi to unconditioned air, resulting in loss of heat and water. That study also confirmed that this stimulus (exposure of these airways to unconditioned air) was associated with damage to the airway mucosa, a feature also reported in humans who exercised while breathing cold air. Our initial investigations into the effects of cold air on inflammatory pathways in the airways of horses serendipitously identified a clear bias toward cytokine expression of T,2 cells 5 hours after a single exercise episode, which included noticeable upregulation of IL-10 mRNA expression. However,

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>∆C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Difference in C&lt;sub&gt;T&lt;/sub&gt; between target and housekeeping genes</td>
</tr>
<tr>
<td>∆∆C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Difference in ∆C&lt;sub&gt;T&lt;/sub&gt; between control and challenge samples</td>
</tr>
</tbody>
</table>
in contrast to results of similar studies\(^7\) in dogs and humans, we did not detect neutrophil influx into the airways of horses. In the study reported here, we used the same procedures to determine whether neutrophil influx into the airways of horses would develop within 48 hours after challenge exposure to cold air while exercising and to test the hypothesis that cytokine expression induced by exercise while breathing cold air would persist for at least 48 hours.

**Materials and Methods**

**Animals**—Nine adult horses (5 geldings and 4 mares) were housed separately in stalls and fed grass hay supplemented with a commercial grain mix\(^1\) throughout a training period and participation in the study. None of the horses used in the study had a history of chronic airway disease, and all horses were clinically normal, as determined by results of physical examination, auscultation while the horse rebreathed expired gases while wearing a rebreathing bag, and blood gas analysis of a sample of arterial blood obtained from each horse. All experiments were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee.

**Procedures**—Training consisted of walking, trotting, and cantering 3 times weekly on a motorized high-speed treadmill in an indoor climate-controlled facility (ambient temperature, 25°C) for 12 weeks. A single graded exercise test was performed after 12 weeks of training to determine the speed and slope that corresponded to a heart rate of 170 beats/min for each horse, and this speed and slope were used for all subsequent exercise tests.

In accordance with a crossover design, horses were assigned to perform an exercise test while breathing warm (25°C and 35% relative humidity [12.6 g of H\(_2\)O/m\(^3\) of air]) or cold (–5°C and > 95% relative humidity [3.5 g of H\(_2\)O/m\(^3\) of air]) air. There was a minimum 1-week washout period between subsequent exercise tests. Each exercise test consisted of walking for 5 minutes (1.8 m/s and 0° slope), trotting for 5 minutes (4 m/s and 0° slope), and cantering for 5 minutes (6.8 to 9.5 m/s and 2.5° slope), with differences only in the temperature and relative humidity of the inspired air. The cold air was produced by drawing ambient air through a high-volume air chiller connected to a loose-fitting face mask worn by each horse during exercise. To prevent the horses from inhaling unchilled ambient air during exercise, the volume of cold air delivered (3,800 L/min) was in excess of the reported maximal inspiratory rate for horses performing submaximal exercise.\(^9\)

**Collection and preparation of samples**—Twenty-four or 48 hours after completion of an exercise test, horses were sedated by IV administration of a combination of xylazine hydrochloride (0.5 mg/kg) and butorphanol tartrate (0.025 mg/kg) and bronchoalveolar lavage was performed by use of a cuffed tube and 240 mL of warmed Hanks’ phosphate-buffered saline solution. The recovered BALF was pooled to determine overall recovery volume, and a 3-mL aliquot was then removed and used for determination of total and differential cell counts. Separate exercise tests were used for each lavage to avoid procedure-related artifact. Slides were prepared by use of cytocentrifugation and stained by use of a modified Wright Giemsa stain.

The remaining BALF was centrifuged at 770 × g for 10 minutes and the supernatant removed. Pelleted cells were resuspended in 1 mL of a phenol–guanidine isothiocyanate mixture,\(^b\) transferred to a 2-mL microcentrifuge tube, and homogenized by use of a disposable pestle. An additional 1 mL of phenol–guanidine isothiocyanate mixture was added to the homogenate, and the sample was frozen at –80°C until extraction of RNA.

**Cytokine analysis**—Cytokine mRNA (IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, interferon-\(\gamma\), and TNF-\(\alpha\)) was quantitated by use of a real-time quantitative reverse-transcription PCR assay and species-specific primers and probes, as described elsewhere.\(^\text{a}\) Quantification of gene amplification was conducted after reverse transcription–PCR assay by determining the C\(_T\) value for indicator fluorescence within the geometric region of the semilogarithmic plot generated during the PCR assay. Within this region of the amplification curve, each difference of 1 cycle is equivalent to a doubling of the amplified PCR product. The relative quantification of target gene expression among treatments was evaluated by use of the comparative C\(_T\) method.\(^\text{10}\) The ΔC\(_T\) value was determined by subtracting the ribosomal C\(_T\) value for each sample from the target C\(_T\) value of that sample. Calculation of ΔΔC\(_T\) for each cytokine and each horse involved the use of the ΔC\(_T\) value of the sample obtained after exercising while breathing warm air as a constant to subtract from the values obtained after exercising while breathing cold air. Thus, the resulting value represents the effect of cold inspired air adjusted for the effect of exercise in general. Fold changes in the relative gene expression of the target were determined by evaluating the expression, \(2^{-\Delta\Delta C_{T}}\).

**Statistical analysis**—Total and differential nucleated cell concentrations in BALF recovered at each time point were compared by use of a paired Student t test. Relative values for cytokine gene expression were not
normally distributed (Kolmogorov-Smirnov; \( P < 0.05 \)) and therefore were analyzed by use of the Wilcoxon signed rank test with a theoretic median value of 1 (no difference from results when exercising while breathing warm air or \( \Delta A_{v} = 0 \)). In all cases, values of \( P < 0.05 \) were considered significant.

**Results**

The proportion of neutrophils in the BALF total nucleated cells was significantly \( (P = 0.027) \) higher 24 hours after exercising while breathing cold air, compared with the proportion 24 hours after exercising while breathing warm air (Figure 1). The BALF neutrophil concentration 24 hours after exercise did not differ significantly \( (P = 0.064) \) between the 2 exercise conditions (Table 1). No other cell type was significantly different in proportion or concentration at either time point between the 2 air temperatures. Total recovery of BALF fluid recovery and total nucleated cell concentrations were not affected by inspired air temperature at 24 or 48 hours after exercise.

Messenger RNA expression of cytokines characteristic of a T\(_{12}\) phenotype was significantly increased \( (P = 0.01) \) 24 hours after exercising while breathing cold air, compared with results 24 hours after exercising while breathing warm air (Figure 2). Expression of T\(_{1}\)-associated cytokine mRNA \( (IL-2\) and interferon-\( \gamma) \) 24 hours after exercise was not altered by the temperature of the air inspired during exercise. Expression of mRNA for general proinflammatory cytokines was upregulated \( (IL-1, 4.4\text{-fold increase}; IL-6, 1.7\text{-fold increase}; and IL-8, 33.3\text{-fold increase}) \) at 24 hours after exercising while breathing cold air. Forty-eight hours after exercise, only IL-10 \( (3.7\text{-fold increase}) \), TNF-\( \alpha \) \( (2.1\text{-fold difference}) \), and IL-6 \( (1.3\text{-fold difference}) \) were significantly different between exercising while breathing cold or warm air.

**Discussion**

Heat and water vapor are transferred from the respiratory mucosa to inspired air during inhalation in a passive process that is driven by physical gradients; this process proceeds until equilibration is achieved. During tidal breathing in resting animals in temperate conditions, the overall demand for transfer of heat and water vapor is relatively low and inspired air equilibrates at body temperature and is completely humidified in the nasal passages. However, during periods of increased ventilation, cold conditions (such as during exercise in cold weather), or both, the combination of the increased volume of air that needs to be warmed and hu-

Table 1—Mean ± SD results for BALF obtained from 9 clinically normal horses 24 or 48 hours after exercising while breathing cold \((-5^\circ C)\) or warm \((25^\circ C)\) air.

<table>
<thead>
<tr>
<th>Time point (hours after exercise)</th>
<th>Air temperature</th>
<th>Volume recovered (mL)</th>
<th>Total nucleated cells (cells/( \mu L ))</th>
<th>Macrophages (cells/( \mu L ))</th>
<th>Lymphocytes (cells/( \mu L ))</th>
<th>Neutrophils (cells/( \mu L ))</th>
<th>Eosinophils (cells/( \mu L ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Warm</td>
<td>130.8 ± 25.7</td>
<td>630.8 ± 255.9</td>
<td>291.5 ± 135.6</td>
<td>326.8 ± 132.3</td>
<td>11.0 ± 5.6</td>
<td>1.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Cold</td>
<td>139.1 ± 19.6</td>
<td>548.9 ± 215.7</td>
<td>251.7 ± 111.4</td>
<td>281.2 ± 125.2</td>
<td>15.9 ± 8.2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>48</td>
<td>Warm</td>
<td>127.5 ± 20.0</td>
<td>446.6 ± 154.0</td>
<td>244.2 ± 60.2</td>
<td>221.7 ± 90.4</td>
<td>6.4 ± 4.7</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Cold</td>
<td>135.0 ± 15.6</td>
<td>366.8 ± 164.6</td>
<td>214.4 ± 115.1</td>
<td>164.9 ± 79.5</td>
<td>7.1 ± 8.0</td>
<td>0.5 ± 0.7</td>
</tr>
</tbody>
</table>

![Figure 2—Box-and-whisker plots of the expression of cytokine mRNA measured in BALF obtained from 9 clinically normal horses 24 (A) or 48 (B) hours after exercising while breathing cold \((-5^\circ C)\) air. Data are the relative increase of cytokine expression after exercising while breathing cold air, compared with expression after exercising while breathing warm \((25^\circ C)\) air. Each box represents the interquartile range, the whiskers represent the range, and the horizontal line within each box represents the median. The relative expression after exercising while breathing warm air was assigned a value of 1 (horizontal line). *Within a cytokine, the median value differs significantly \( P < 0.05 \) from a value of 1. IFN = Interferon.](image)
ture of these responses in mammals and further validating horses for use in evaluating cold air–induced airway damage in humans.

The temporal pattern of responses in horses following exposure of peripheral airways to unconditioned air differs significantly from those in dogs and humans. In the latter species, neutrophil recruitment is evident within 5 hours after challenge exposure, whereas in horses, this response required between 5 and 24 hours to develop (Figure 1). We do not believe that the difference between the responses for these species is a result of the magnitude of the initial challenge exposure because even small exposures are capable of inducing neutrophil influx in airways of dogs. Rather, it is possible that the multiple steps leading from challenge exposure of airways with unconditioned air to neutrophil influx proceed more slowly in horses, compared with the rate in other species. If this is true, then the prolonged developmental phase of the response to cold air may be more amenable to pharmacologic intervention.

We detected important differences between cytokine expression in the study reported here and that reported in another study in which BALF was collected 5 hours after challenge exposure. In that other study, the most prominent changes were substantial increases in the expression of the T helper 2 cytokines IL-4, IL-5, and IL-10, whereas IL-2 and IL-6 expression were also significantly increased, but the increases were more modest. Between 5 and 24 hours after challenge exposure, the overall pattern shifts to one more characteristic of generalized inflammation, with more substantial increases in IL-1, IL-6, and IL-8 expression and reduced expression (compared with the value 5 hours after challenge exposure) of at least 2 of the T helper 2 cytokines (IL-4 and IL-5) by 24 hours after challenge exposure. By 48 hours after challenge exposure with cold air, expression of most cytokines is not different from that detected 48 hours after challenge exposure with warm air, with significant but quantitatively small increases in TNF-α and IL-6 expression. Only IL-10 expression remained substantially increased 48 hours after exposure to cold air.

We are unable to ascribe specific consequences to most of the changes in cytokine expression reported here and in the other study. However, it is likely that IL-8, a neutrophil chemoattractant, is responsible for the increased percentage of neutrophils in the BALF found 24 hours after challenge exposure with cold air. It is important to mention that in the study reported here and another study conducted by our laboratory group, we measured cytokine mRNA and not the actual protein product, thus leaving open the possibility for smaller changes in actual protein product as a result of posttranslational regulation. However, there is circumstantial evidence that the increased expression of IL-10 has important consequences, such as causing an increased susceptibility of the respiratory tract to viral pathogens.

The changes in airways of horses in response to exposure to cold air are modest, compared to those for humans. We believe that the muted responses in horses are a specific product of the experimental design and not necessarily qualitatively representative of the athletic horse population in general. First, to study de novo induction of cold air–induced airway injury, we meticulously managed the horses used in our studies to minimize preexisting airway inflammation by reducing exposure to dusts, unfamiliar horses, and cold air (achieved by conducting the training and exercise activities during the summer months in warm conditions). Furthermore, by limiting the exercise test to an amount of activity that causes a heart rate of 170 beats/min (substantially lower than the maximal heart rate), we restricted the intensity of the exercise tests to a magnitude substantially less than the maximal exercise capacity of horses to avoid the potentially confounding effects of exercise-induced pulmonary hemorrhage on BALF cytologic examinations. As a result, the increase in minute ventilation during our exercise tests is less than that during maximal exercise. Because the magnitude of the loss of heat and water vapor during exercise and the subsequent inflammatory response are both a function of increased ventilation, it is reasonable to expect that horses performing more strenuous exercise would have greater injury to the airway mucosa and subsequent airway obstruction and inflammation when exercising in subfreezing conditions.

Similarly, because the magnitude of airway cooling (and resulting airway injury) is also a function of the temperature of the inspired air, it is reasonable to expect that airway disease comparable to that in the horses of our studies (moderate increase in ventilation and extremely cold inspired air) could be induced in maximally exercising horses while breathing warmer air. In that regard, it is important to mention that a similar degree of damage to the airway mucosa (as measured by recovery of ciliated epithelial cells) was detected in racehorses after strenuous workouts conducted in ambient conditions at temperatures > 0°C.

Modest changes in airway neutrophils induced by exposure to cold air in the study reported here may underestimate the responses found in typical equine athletes. Similar concentrations of BALF neutrophils were reported after a single challenge exposure in dogs by use of a wedged bronchoscope method to simulate exercise while breathing cold air, and more importantly, substantially higher concentrations were reported after repeated challenge exposures (the scenario more relevant to an athlete in training). Thus, it is likely that repeated challenge exposure of airways of horses with unconditioned air would induce a greater influx of neutrophils.

Finally, it is important to consider that the airway responses of horses to cold air reported here and in other studies conducted by our laboratory group are responses of normal airways. The aggregate literature on responses of humans to cold air suggests that although moderate responses can be elicited from clinically normal humans, humans with preexisting airway inflammation have exaggerated responses and repeated challenge exposures can induce chronic airway inflammation in humans. These observations may be directly applicable to the racehorses, a population that has a high prevalence of airway inflammation and that performs repeated bouts of strenuous exercise in the course of training and competition. Thus, exposure
of bronchi and bronchioles to cold air may be an important cause of chronic airway inflammation in racehorses that are training or racing in cool climates.

The consequences of airway exposure to cold air may not be limited to the induction of neutrophilic inflammation but may also include increased susceptibility to viral pathogens. Many investigators have compiled data supporting the concept of the open window of transient immune suppression following strenuous exercise. Strenuous exercise results in systemic immunosuppression and changes in systemic cytokine production, with the latter effect possibly mediated through the effects of exercise-induced cortisol secretion. In contrast, moderate exercise may actually protect against respiratory tract infections in humans. Horses have an increased susceptibility to influenza following strenuous exercise, and the authors of that report suggested that the increased susceptibility was the result of systemic immunosuppression. The exercise tests used in the study reported here, and the systemic effects attributable to those tests, could be considered moderate relative to the overall exercise capacity of the horses, but the experimental design provided for challenge exposure of the airways with unconditioned air typical of maximal exercise while specifically decreasing the likelihood of systemic contribution to the local airway responses.

Thus, although we cannot exclude a systemic contribution to exercise-induced immunosuppression during strenuous exercise, analysis of our data raises the additional possibility of local suppression of cell-mediated immunity in the respiratory tract through the prolonged expression of IL-10 in the airways as a direct result of breathing cold air during exercise. One potential deleterious effect of increased IL-10 expression is increased susceptibility to viruses, such as influenza viruses, through impaired production of type I interferons. Furthermore, increased concentrations of IL-10 at 24 and 48 hours after exercise indicate that compromised immunity to viruses may persist well after conclusion of exercise (Figure 2). The apparent capacity of a single episode of exercise while breathing cold air to induce prolonged suppression of cell-mediated immunity in the respiratory tract may be a major factor in the pattern of influenza outbreaks during the winter months. Further evaluation of the role of cold air in local immune function in the airways may serve a critical role for use in developing more effective strategies to decrease susceptibility to influenza and reduce the severity of annual outbreaks.

References