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Bronchoalveolar Lavage Fluid Cytology and Cytokine Messenger Ribonucleic Acid Expression of Racehorses with Exercise Intolerance and Lower Airway Inflammation

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Background: There is limited information relating bronchoalveolar lavage (BAL) cytology and cytokine messenger ribonucleic acid (mRNA) expression in racehorses with inflammatory airway disease (IAD).

Hypothesis and Objective: We hypothesize that cytokine expression in BAL cells would correlate with cytology. Thus, we evaluated the mRNA expression of selected cytokines in BAL cells in racehorses with exercise intolerance and lower airway inflammation.

Animals: Thirty-one client-owned Standardbred racehorses with exercise intolerance.

Methods: Prospective, observational study. Cells were obtained by BAL, and mRNA expression of interleukin (IL)-1β, IL-4, IL-8, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results: Nine horses had normal BAL cell differential cytology (Controls), while 22 horses had evidence of IAD based on BAL fluid cytology. Relative expressions of TNF-α/glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 0.0092 ± 0.010 versus 0.0045 ± 0.005, $P = .034$), IL-4/GAPDH (0.001 ± 0.002 versus 0.0003 ± 0.0003, $P = .029$), and IFN-γ/GAPDH (0.0027 ± 0.003 versus 0.0009 ± 0.001, $P = .028$) were greater in horses with IAD compared with controls. Furthermore, IL-4/GAPDH (0.001 ± 0.002 versus 0.0002 ± 0.0003, $P < .0001$) and IFN-γ/GAPDH (0.003 ± 0.003 versus 0.001 ± 0.001, $P = .002$) mRNA expression was increased in horses with increased metachromatic cell counts compared with horses with normal metachromatic cell counts. Only the mRNA expression of IL-1β/GAPDH (1.1 ± 0.7 versus 0.3 ± 0.3, $P = .045$) was increased with airway neutrophilia.

Conclusions and Clinical Importance: Differences in gene expression were associated with the presence of IAD and with specific cell types present in airway secretions of Standardbred racehorses with poor performance. These findings suggest that different pathophysiological pathways are implicated in IAD.

Key words: Inflammatory airway disease; Interferon-γ; Interleukin-4; Tumor necrosis factor-α.

Inflammatory airway disease (IAD) is defined as a condition of horses associated with exercise intolerance, or coughing combined with nonseptic pulmonary inflammation or dysfunction.[1] Conditions leading to lower airway inflammation are numerous and the etiopathogenesis of IAD is currently unknown. Heaves, a condition associated with severe airway inflammation, results from exposure to dust particles present in hay feeding and straw bedding. A similar process also appears to contribute to IAD as stabling of both young and adult horses otherwise free of respiratory diseases results in lower airway inflammation.[2,3] However, while heaves-susceptible horses put in the offending environment will develop persistent airway obstruction and inflammation, IAD is transient in most horses.[4] It has been hypothesized that long standing IAD could precede the development of heaves, but there are currently no markers shown to detect horses that will eventually progress toward heaves.

Abbreviations:

EIPH	exercised-induced pulmonary hemorrhage
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IAD	inflammatory airway disease
IFN	interferon
IL	interleukin
mRNA	messenger ribonucleic acid
PMNs	polymorphonuclear leukocytes
RT-qPCR	reverse transcription quantitative polymerase chain reaction
Th	T-helper cells
TNF	tumor necrosis factor

A heterogeneous cell population can be observed in bronchoalveolar lavages (BAL) of horses with IAD; mast cells, eosinophils, or neutrophils might be detected in increased numbers.[1] The inflammatory cell population present appears to be relevant, as clinical signs or lung function abnormalities have been linked to specific cell types in horses. For instance, increased BAL mast cells[5] and eosinophils[6] have been associated with airways hyperresponsiveness, while horses with airway neutrophilia tended to be older and had more prominent clinical signs of respiratory diseases.[5] Whether the predominance of a given cell population represents a distinct pathophysiological process or various stages of the disease is currently unknown. Interestingly, eosinophils are more common in BAL from human patients with mild to moderate asthma[7] whereas neutrophils, the predominant cells in the airways in heaves, are frequently observed in severe asthmatics.[8]

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Cytokines are mediators contributing to the regulation of the immune response and the networking of neighboring or distant cells. They are produced by all cell types, and are implicated in initiation, maintenance, and resolution of inflammation. The study of their expression serves to understand the pathophysiological events implicated in disease processes and might be used as signatures for the diagnosis of specific diseases. We hypothesize that cytokine messenger ribonucleic acid (mRNA) expression in BAL fluid cells would correlate with cytology and reflect different pathological phenomenons. Thus, we evaluated the mRNA expression of interleukin (IL)-4, interferon (IFN)-γ, IL-8, IL-1β, and tumor necrosis factor (TNF)-α in BAL cells in a group of racehorses presented with a history of exercise intolerance. These cytokines were chosen because they have been associated with specific disease processes and with pulmonary inflammation in heaves.[9–16]

Material and Methods

Thirty-one Standardbred racehorses with exercise intolerance were studied. Horses were 4.16 ± 2.2 years old (range 2–10 years), and there were 13 mares, 12 geldings, and 6 stallions. All horses had presented a decreased in performance as determined by both the trainer and the referring veterinarian, in the last 2 races. All horses had been trained the day before examination (12–30 hours previously) at their usual racing speed. No horses were found to be lame when trotting on a straight line.

Clinical Evaluation

Evaluation of horses upon admission at the veterinary teaching hospital of the Université de Montréal included a complete physical examination with a thoracic auscultation using a rebreathing bag, a CBC, and determination of plasma AST and CK activities.

Endoscopy of the Upper Airways

Videoendoscopic evaluation of the airways, including the guttural pouches and trachea, was performed.

BAL

BAL were performed as described previously.[17] In brief, horses were sedated with xylazine[a] (0.6–1.0 mg/kg IV) and butorphanol[b] (20–30 μg/kg IV). A flexible videoendoscope (length 2.5 m, external diameters 10.5 mm)[c] was passed through the nares and directed down into the right lung until its tip was wedged in the wall of a bronchus. Diluted (0.5%) lidocaine[d] was used to desensitize the airway mucosa. Two 250 mL boluses of prewarmed sterile isotonic saline (37°C) solution were rapidly instilled into the bronchus and aspirated via the endoscope's biopsy specimen channel. Samples were stored in an ice slurry and analyzed within 1 hour of collection. Total nucleated cells in BAL fluid samples were counted with a hemocytometer. Smears of the fluid were prepared by centrifugation (at 100 × *g* for 5 minutes) and stained with a modified Wright's solution.[e] Differential counts were made on 400 cells excluding epithelial cells. The fluid was filtered through sterile gauze centrifuged at 4°C and washed twice in PBS. Cell viability was assessed by Trypan blue dye exclusion test. Two aliquots of 10^7 cells were homogenized in TRIzol Reagent[f] and immediately frozen at −80°C for RNA extraction.

RNA Extraction and Reverse Transcription (RT)

RNA extraction was performed according to the manufacturer instructions[f] by 3-step nucleic acid precipitation with 0.2 volume chloroform, 1 volume isopropanol, and ethanol. RNA pellets were air-dried and total RNA concentration and purity was evaluated by spectrophotometry.[g]

The 260/280 absorbance ratios ranged between 1.51 and 2.0 (mean 1.83). All RNA samples were used for RT and quantitative polymerase chain reaction (qPCR). Two micrograms of total RNA in 9 μL of RNAse-DNase free water[h] was heated at 70°C for 5 minute[i] for RT. One microliter of oligo$(dT)_{12-18}$[j] primers was added and the mixture was heated for 10 minute at 70°C. Samples were heated at 50°C for 1 hour after adding 20 μL of a mastermix containing 0.01 M Dithiothreitol,[j] 1.6 mM dNTP,[j] 2 U/μL of RNA Inhibitor,[k] and 1.2 U/μL AMV reverse transcriptase[l] reaction was stopped by heating the samples at 100°C for 1 minute. Samples were stored at −20°C.

qPCR

Real-time PCR was performed with Quantitect SYBR Green PCR kit[m] according to the manufacturer's protocol with minor modifications on RotorGene thermal cycler.[n] Briefly, 1 μL of cDNA was added to obtain 20 μL final reaction volume containing 0.5 μM of each sense and antisense primers (see Table 1 for primer sequences) and 2.75 mM $MgCl_2$. Amplification conditions were 10 minute at 95°C followed by 40 cycles of amplification cycles. For each gene, a serial dilution (10×) of known concentration of PCR products[o] (ng/μL) was performed to obtain a standard curve, which was optimized in order to give reproducible efficiency coefficient (>0.90). The absolute concentration of each gene was obtained in cDNA samples using imported standard curves and adjustment to a standard curve reference included in each run. Relative gene expression was calculated using the ratio of the target gene absolute concentration (ng/μL) divided by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or CD3ζ absolute concentration (reference genes) within each sample. Cytokine primers were designed to span exon-intron boundaries in order to prevent amplification of genomic DNA.

Table 1. Primer pairs used for quantitative polymerase chain reaction

	Sense (5′ → 3′)	Antisense (5′ → 3′)
CD3ζ	GCA TCG TAG GTG TCC TTG GT	AGA GGA GTATGA CGG CAT CG
GAPDH	AAG TGG ATA TTG TCG CCA TCA AT	AAC TTG CCA TGG GTG GAA TC
IL-1β	GAC TGA CAA GAT ACC TGT GGC CT	AGA CAA CAG TGA AGT GCA GCC T
IL-4	TCG TGC ATG GAG CTG ACT GTA	GCC CTG CAG ATT TCC TTT CC
IL-8	CTT TCT GCA GCT CTG TGT GAA G	GCA GAC CTC AGC TCC GTT GAC
INF-γ	TCT TTA ACA GCA GCA CCA GCA A	GCG CTG GAC CTT CAG ATC AT
TNF-α	CTT GTG CCT CAG CCT CTT CTC CTT C	CTTGTGCCTCAGCCTCTTCTCCTTC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; INF, interferon; TNF, tumor necrosis factor.

Statistical Analysis

Differences between groups in cytokine expressions were evaluated by unilateral Student's *t*-tests with Welch's correction for unequal variances because we were making a prediction about the direction of the effect.[P] One-way analysis of variance and Dunnett's multiple comparison test or bilateral *t*-test were used for other parameters. Data were log 10 transformed to normalize distributions when appropriate. Correlations were performed by Pearson's product-moment correlation coefficient. Data are presented as mean±SD, unless stated otherwise. A difference of $P \leq .05$ was considered significant.

Results

Clinical, Hematological, and CK and AST Findings

No cough was reported in 17 horses, while 5 and 2 horses had occasional or frequent coughing episodes, respectively. Physical examination revealed no significant abnormal findings other than the presence of wheezes auscultated with a rebreathing bag in 1 horse. CBCs were within reference ranges in 17 of 19 horses; 1 horse had a polycythemia (PCV, 57%, normal 32–52%) and another had a neutrophilia ($9.0 \times 10^3/\mu L$, reference range 2.7–6.7 $\times 10^3/\mu L$). These changes were believed to be stress related. The median plasma CK and AST values were 167 μ/L (range 98–1422 μ/L) and 313 μ/L (range 202–2096 μ/L), respectively. Three horses had values of AST above the maximal reference range value of 500 μ/L (range 501–2096 μ/L) and of 350 μ/L for the CK (662–1422 μ/L), while 3 additional horses had increased AST values only (range 561–854 μ/L). The increased muscle enzymes in these horses were not considered the cause of exercise intolerance by the attending clinician.

Endoscopy of the Upper Airways

Important findings included the presence of an ulcer on the free border of the soft palate in 1 horse. Most horses had variable degrees of nodular lymphoid pharyngitis.

BAL Fluid Cytology

Twenty-seven out of 28 horses in which this information was noted had exercised-induced pulmonary hemorrhage (EIPH) based on the presence of hemosiderophages on BAL fluid cytology or the presence of red tinged BAL fluid. Nine horses had normal BAL fluid cell differential cytology (polymorphonuclear leukocytes [PMNs] ≤5%, metachromatic cells <2%, and eosinophils <1%) and were defined as controls. The remaining 22 horses had variable combinations of abnormal cytology. Fourteen horses had ≥2% metachromatic cells (range 2–4%) in BAL, 5 of which also had >5% neutrophils, and 3 horses had ≥1% eosinophils. Of the 11 horses with >5% neutrophils (range 6–19%) in BAL, 5 horses also had increased metachromatic cell percentages (≥2%), with 2 horses having both increased metachromatic cells and eosinophils.

The volume of BAL liquid recovered was significantly ($P = .03$) smaller in horses with increased metachromatic cells (274 ± 54 mL) than in other horses (327 ± 92 mL).

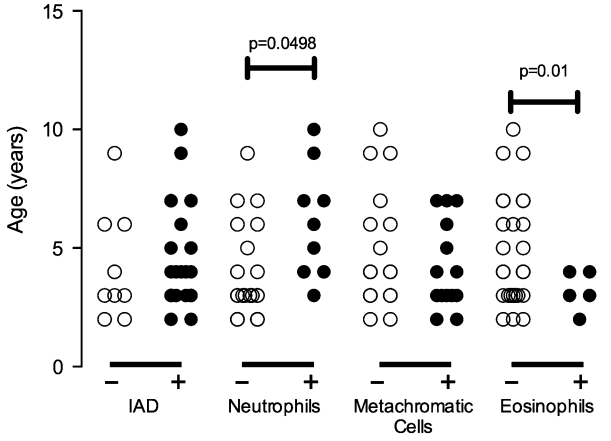

Fig 1. Age (years) of Standardbred racehorses when divided based on bronchoalveolar lavage fluid cytology into horses without (–, open circles) or with (+, black circles) inflammatory airway disease (IAD), increased neutrophils, metachromatic cells, or eosinophils.

The total cell number in BALs of horses with airway neutrophilia ($66 \pm 43 \times 10^6$) was increased ($P = .004$) when compared with other horses ($22 \pm 23 \times 10^6$). These horses were also older (5.7 ± 2.9 years; Fig 1) than other horses with IAD (3.8 ± 1.8 years, $P = .042$) or than all horses without airway neutrophilia (including controls, 4.0 ± 2.0 years, $P = .049$). Horses with airway eosinophilia (3.2 ± 0.8) were younger ($P = .01$) than other horses (4.9 ± 2.3). In the 7 horses reported to cough by the owner/trainer, 6 had abnormal cytology. The only horse with abnormal lung auscultation had normal BAL fluid cytology.

Cytokine mRNA Expression

GAPDH and CD3ζ coefficient of variation among samples was 1.02 and 1.23, respectively, and their expression did not differ significantly between cases and controls. The mRNA expression of TNF-α/GAPDH ($P = .034$), IL-4/GAPDH ($P = .029$), and IFN-γ/GAPDH ($P = .028$) was significantly increased in horses with IAD when compared with controls based on BAL fluid cytology (Fig 2A). Gene expression was also marginally increased for IL-1β/GAPDH ($P = .0550$) and IL-8/GAPDH ($P = .085$). Expression of IL-4 ($P < .0001$) and IFN-γ ($P = .002$) were significantly increased in horses with increased metachromatic cells when values were corrected for GAPDH (Fig 2B) but not with CD3ζ. Only the expression of IL-1β ($P = .045$) was significantly increased with BAL neutrophilia (Fig 2C) and no differences in gene expression were observed with eosinophilia. ANOVA also identified significant differences in IFN-γ/GAPDH, IL-4/GAPDH, and IL-1β /GAPDH mRNA expression in the control horses and in a subset of horses presenting only increased metachromatic cells (n = 8), or neutrophils (n = 6). This was because of an increased expression of IFN-γ/GAPDH (Fig 3A) and IL-4/GAPDH (Fig 3B) in horses with increased metachromatic cells when compared with controls and in increased IL-1β/GAPDH expression in horses with airway neutrophilia (Fig 3D).

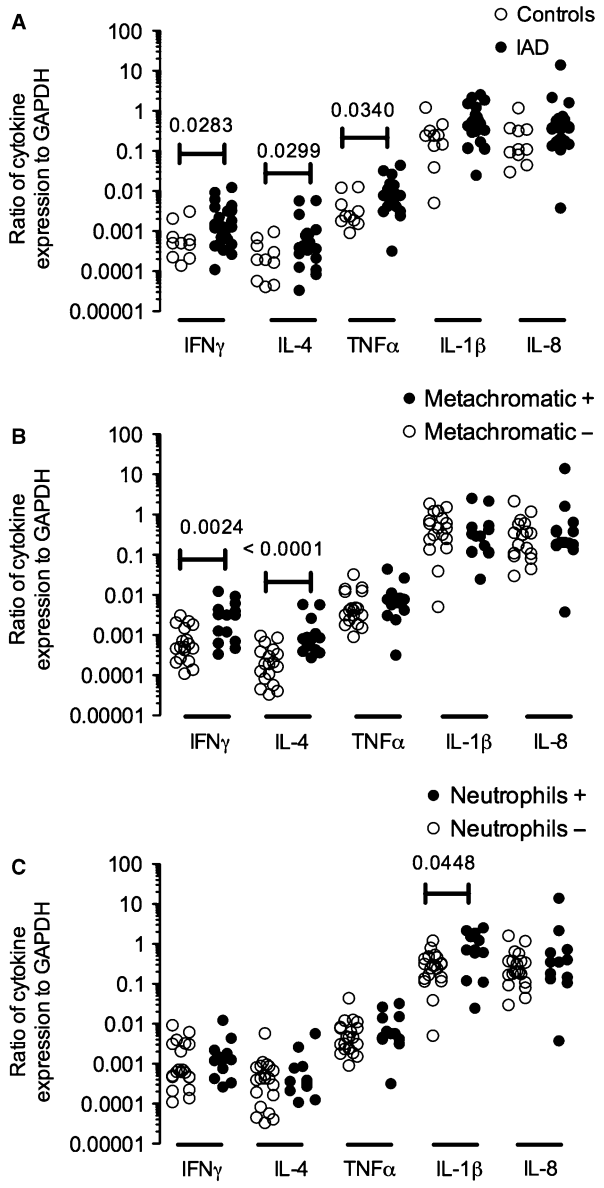

Fig 2. Relative expression of interferon (IFN)-γ, interleukin (IL)-4, tumor necrosis factor (TNF)-α, IL-1β, and IL-8 messenger ribosomal nucleic acid (mRNA) by bronchoalveolar lavage (BAL) cells when horses are divided based on BAL fluid cytology as (**A**) controls (polymorphonuclear leukocytes [PMNs] ≤5%, metachromatic cells <2%, and eosinophils <1%) and IAD; (**B**) normal (metachromatic −, <2%) or increased (metachromatic +) metachromatic cell counts; (**C**) normal (neutrophils −, <5%) or increased (neutrophils +) neutrophils cell counts. Results are expressed as a ratio of expression of studied genes to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a reference gene.

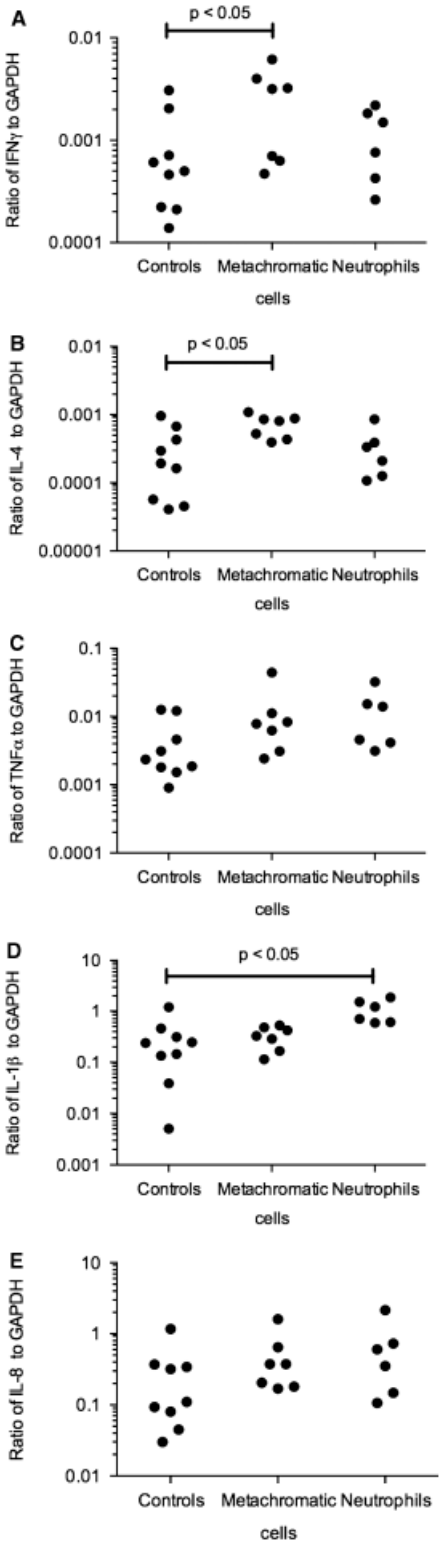

Fig 3. Relative expression of interferon (IFN)-γ (**A**), interleukin (IL)-4 (**B**), tumor necrosis factor (TNF)-α (**C**), IL-1β (**D**), and IL-8 (**E**) messenger ribosomal nucleic acid (mRNA) by bronchoalveolar lavage (BAL) cells of horses with normal BAL fluid cytology (controls, polymorphonuclear leukocytes [PMNs] ≤5%, metachromatic cells <2 %, and eosinophils <1%; n = 9) or with increased metachromatic cells (≥2%; n = 8) or neutrophils (>5%; n = 6). Results are expressed as a ratio of expression of studied genes to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a reference gene.

Correlations

Percentages and total numbers of metachromatic cells in BAL were positively correlated with IL-4/GAPDH (Pearson's $r = 0.62$, $P = .0009$ and Pearson's $r = 0.64$, $P < .0001$, respectively), IL-4/CD3ζ (Pearson's $r = 0.51$, $P = .009$ and Pearson's $r = 0.44$, $P = .01$, respectively), and IFN-γ/GAPDH (Pearson's $r = 0.61$, $P = .001$ and

Pearson's $r = 0.44$, $P = .01$, respectively) mRNA expressions, but not with IFN-γ/CD3ζ (Pearson's $r = 0.32$, $P = .11$ and Pearson's $r = 0.14$, $P = .46$, respectively). IL-1β / GAPDH expression was positively correlated with the percentage of neutrophils in BAL (Pearson's $r = 0.56$, $P = .001$) but not with the total number of neutrophils (Pearson's $r = 0.33$, $P = .07$).

Discussion

Multiple pathways could lead to lower airway inflammation, and the study of immunologic mechanisms implicated in IAD is considered a research priority of this syndrome in a recent ACVIM consensus statement.[1] In the present study TNF-α, IL-4, and IFN-γ mRNA were increased in BAL of horses with IAD, while expression of IL-4 and IFN-γ mRNA was also associated with increased metachromatic cells. Only increased IL-1β mRNA expression was associated with airway neutrophilia. These distinctive cytokine signatures suggest that several pathophysiological disease processes are implicated in IAD.

BAL Fluid Cytology

More than half (14/22) of horses with IAD herein had increased metachromatic cells in BAL, and airway eosinophilia was present in 5 horses. We used the term "metachromatic cells" as both mast cells and basophils are increased in the airways of asthmatic patients[18] and metachromatic staining does not allow to differentiate these 2 cell populations. Thus, both of these cell populations could possibly be present in IAD. The high prevalence of metachromatic cells we observed is of interest as airway hyperresponsiveness in horses is significantly associated with increased BAL mast cells[5] or eosinophil counts,[6] but not with neutrophilic airway inflammation (BAL neutrophils $>5\%$). In Standardbred racehorses, both BAL fluid eosinophil and mast cell counts were significantly correlated with abnormal lung function,[19] although no distinct clinical signs were noted in horses despite marked pulmonary eosinophilia (range 1–37%, median 2%).[20] The decreased volume of BAL fluid recovered in horses with increased metachromatic cells we observed could further provide evidence of the presence of airway hyperresponsiveness in these horses.

Neutrophils are the predominant BAL cell population in heaves, but a variable subset of horses with IAD have increased neutrophils in their airways.[1] In agreement with a previous report,[21] horses herein had only mild airway neutrophilia (6–19%), when compared with values observed in heaves ($>25\%$).[22] This finding would be expected as high percentages of BAL neutrophils are associated with coughing episodes,[5] and most horses studied here had no overt clinical signs of airway disease. Of interest, and as observed previously,[5] horses with increased BAL neutrophils were significantly older than those with other cytological abnormalities. Whether the increased prevalence of airway neutrophilia with age represents a different pathophysiological process leading to airway inflammation than those responsible for the presence of mast cells or eosinophils in IAD is unknown. However, as the severe airway obstruction observed with heaves is associated with airway neutrophilia and preferentially affects older horses, it is tempting to postulate that processes leading to mast cell or eosinophil accumulation in the airways are either self-limiting, or that neutrophilic inflammation represents a maturation process, as suggested previously by Hare and Viel.[6]

IAD and Exercise Intolerance

Standardbred racehorses with exercise intolerance and lower airway inflammation, but with normal respiratory mechanics at rest, were reported to have increased lung resistance and frequency-dependent lung compliance during hyperinflation.[23] Similarly, airway obstruction was observed by impulse oscillometry, a noninvasive and sensitive lung function test, in a group of Standardbred racehorses with IAD but without other clinical signs of respiratory disease.[19] Horses from the present study had a history of exercise intolerance believed to be caused by upper airway obstruction, because no lameness or overt signs of respiratory disease were observed. Interestingly, 68% of these horses had lower airway inflammation. Taken together, these results suggest that IAD is likely to be underdiagnosed in racehorses, as clinical signs suggestive of lower airway inflammation are often absent.

Th1/Th2 Profiles

$CD4^+$ lymphocytes, also known as T-helper cells, subsets type 1 (Th1) and type 2 (Th2) have been shown to orchestrate inflammation in a number of animal and human pulmonary diseases. IL-4 and IFN-γ are archetypical cytokines for Th2- and Th1-type responses, respectively. Overexpression of Th2 cytokines leads to an allergic response while Th1-type cytokines could cause uncontrolled tissue damage. Th1/Th2 responses often coexist but in predominant proportions in natural human and animal diseases.

In the present study, both IL-4 and IFN-γ mRNA were significantly overexpressed in horses with increased metachromatic cells. Increased IL-4 has also been associated with heaves[9–11] and SPAOPD,[12] although this finding is not universal.[13] Cells that have been reported to express IL-4 include basophils, mast cells, eosinophils, and lymphocytes.[11,24] As the cell population responsible for the increased expression of IL-4 by RT-PCR cannot be ascertained, results of the present study could either represent a predominant Th2-cell response, or merely reflect the increased metachromatic cell population in the airway lumen in these horses. As the expression of IL-4 was not significant when corrected with CD3ζ, a specific marker for lymphocytes use to correct for the variation of T-cell numbers in BAL,[25] but was correlated with metachromatic cells in BAL, the latter cells, rather than lymphocytes, could have possibly contributed to increased IL-4 mRNA expression. Alternatively, each IL-4 producing T-cells could have upregulated the expression of this cytokine.

The increase in IFN-γ in horses with increased metachromatic cells is more surprising. While it was believed that Th1 lymphocytes, cytotoxic lymphocytes, and NK cells exclusively produced IFN-γ, it is now recognized that professional antigen presenting cells such as macrophage and dendritic cells are also an important local source for this cytokine.[26] The positive correlation of IFN-γ to metachromatic cells using GAPDH but not CD3ζ, possibly suggests that a source other than lymphocytes are responsible for the increase in IFN-γ. Nevertheless, increased IFN-γ alone or in association with an increase in IL-4 has also been observed in BAL cells in heaves[10,13] or SPAOPD[12,14] by RT-PCR. These results suggest that a common pathway perhaps contributes to mild and more severe pulmonary inflammation in IAD and heaves, respectively. However, our results contrast with the previous findings that IFN-γ expression is not associated with IAD,[27] a finding possibly reflecting the multiple disease phenotypes encompassed by the current IAD definition.

IL-8 mRNA Expression

Increased expression of IL-8, the most potent chemokine for neutrophils, has been consistently linked to the airway neutrophilia in horses with heaves.[10,13,15,16] In the present study, IL-8 mRNA expression was not significantly increased when comparing horses with neutrophilia versus controls. Expression of IL-8 by epithelial cells is reported to be increased in heaves,[16,28] and inflammatory cells such as macrophages[29,30] and even neutrophils[31] are also likely to be important sources of this cytokine when studying BAL cells. Our findings suggest that other cell types such as epithelial cells or mediators others than IL-8 contributed to airway neutrophilia in IAD horses. It could also possibly reflect the lack of specificity of qPCR when studying mixed cell populations as discussed below.

TNF-α and IL-1β

IL-1β and TNF-α are cytokines secreted by many cell types, primarily by macrophages, that modulate inflammatory and immune response. IL-1β is not present in homeostatic conditions and together with TNF-α, initiates and amplifies the inflammatory responses (reviewed by [32]). Lower airway inflammation was associated with an increased expression of TNF-α in horses studied here, and airway neutrophilia was correlated to IL-1β expression. Interestingly, exacerbation of heaves is associated with increased TNF-α and IL-1β expression.[10] The airway epithelium of horses with heaves has an increased expression of TNF-α,[33] and it has been suggested that airway neutrophils and macrophages could also express these cytokines in this disease.[30,34] These cell types are also likely to have contributed to the increased expression of TNF-α and IL-1β observed here.

Limitations of the Present Study

Inclusion criteria, time of sampling, and techniques used to measure cytokine mRNA expression might influence results of studies such as this one. Exercise intolerance was determined by both referring veterinarians and trainers. While their assessment was subjective, it is unlikely that these horses performed as expected, because of the time and cost of transportation to our hospital. Dynamic upper airway obstruction and EIPH could have contributed to exercise intolerance in these horses, although they do not appear to influence BAL fluid cytology in horses with poor performance.[22] Also, horses were subjected to intense exercise (simulating racing conditions) before examination.[21] Exercise has been associated with altered cytokine expression in the blood of human and equine athletes[35,36] but did not influence gene expression in BAL of horses in one study.[37] Cold air exercise has been associated with altered cytokine gene expression in horses,[38,39] and some of these horses were studied during the winter months. However, BAL fluid cytology remained within reference ranges with cold air,[38,39] suggesting that other causes influenced airway cell population in the present study. Concurrent medical conditions or causes of decreased performance that had not been identified during the clinical examination, duration, and condition for the transport of horses to our hospital, are additional factors that could have influenced our results. Lastly, as in most equine reports on the cytokine expression by BAL cells, we used qPCR analysis on a mixed BAL cell population. This technique while very sensitive does not permit the study of cytokine expression by individual cell types. In situ hybridization has been used in equine BAL cells to identify specific cell populations expressing a given cytokine,[9,11] but this method is labor intensive, which limits its use for the study of large cohorts of horses. Studying the protein expression in BAL cells might represent a preferable approach, but is limited by the equine specific reagents currently available.

In summary, distinct cytokine mRNA signatures in airway secretion were associated with the presence of lower airway inflammation in racehorses with exercise intolerance. The cytokine expression profiles varied depending on whether neutrophils or metachromatic cells were present in increased quantity in BAL fluid. However, as all horses studied were exercise intolerant, a possible link between poor performance and cytokine expression in BAL fluid was not evaluated. Future studies should evaluate the cytokine profiles of other IAD phenotypes and their possible associations with performance.

Footnotes

[a] Rompun, Bayvet, Etobicoke, ON, Canada
[b] Torbugesic, Ayerst Laboratories, Montreal, QC, Canada
[c] Olympus Medical Systems Corp, Tokyo, Japan
[d] Lurocaine, Vetoquinol, Lavaltrie, QC, Canada
[e] Hematek, Bayer Diagnostics, Elkhart, IN
[f] Invitrogen, Burlington, ON, Canada
[g] SpectrophotometerGeneQuantpro, Biochrom, Cambridge, UK
[h] GIBCO, Invitrogen
[i] Standard Heatblock, VWR International, Ville Mont-Royal, Canada
[j] Oligo$(dT)_{12–18}$, Invitrogen

[k] RNAguard(Porcine), Amersham Biosciences, GE Lifesciences, Baie d'Urfe, QC, Canada
[l] Roche Diagnostic, Laval, QC, Canada
[m] Quantitect SYBR Green PCR kit, Qiagen, Mississauga, ON, Canada
[n] Rotor-Gene Real-Time Centrifugal DNA Amplification System 3000, Corbett Research, Montreal Biotech, Montreal, QC, Canada
[o] Gel Extraction Kit, Qiagen
[p] Prism v.5.0, La Jolla, CA

Acknowledgments

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