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Research paper

Evaluation of cytokine mRNA expression in bronchoalveolar lavage cells from horses with inflammatory airway disease

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ABSTRACT

Inflammatory airway disease (IAD) is a common disorder of performance horses and is associated with poor performance and accumulation of mucus and inflammatory cells in lower airway secretions. Horses with IAD frequently have increased relative counts of neutrophils in bronchoalveolar lavage fluid (BALF); less commonly relative counts of eosinophils and/or mast cells may be increased. The aetiopathogenesis of IAD is unknown and may involve innate and/or acquired immune responses to various factors including respirable dust constituents, micro-organisms, noxious gases and unconditioned air. The molecular pathways and role of the immune system in the pathogenesis of IAD remain poorly defined and it is unknown whether polarised T cell responses occur in the disease, as have been reported to occur in equine recurrent airway obstruction and asthma in humans. Elucidating cytokine responses that develop in horses with IAD may allow a greater understanding of the possible aetiopathological pathway(s) involved and could contribute to development of novel treatments. We compared the mRNA expression of tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin (IL)-1 β , IL-2, IL-4, IL-8, IL-13, IL-17 and IL-23 in cell pellets extracted from BALF of horses with IAD ($n = 21$) and horses free of respiratory tract disease ($n = 17$). Horses with IAD had significantly increased levels of TNF- α , IL-1 β and IL-23 mRNA; no significant differences in the other cytokine mRNAs were detected. The results of this study indicate that IAD of horses is associated with increased mRNA expression of pro-inflammatory cytokines in BALF cells, which may reflect stimulation of the innate immune responses to inhaled antigens. There was no evidence of a polarised T-cell cytokine response suggesting hypersensitivity responses may not be involved in the aetiopathogenesis of IAD.

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Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CD-3 ξ , cluster determinant 3-zeta; IAD, inflammatory airway disease; mRNA, messenger ribonucleic acid; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RAO, recurrent airway obstruction; RNA, ribonucleic acid.

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1. Introduction

Inflammatory airway disease (IAD) is a common pulmonary disorder of performance horses and is associated with poor performance, accumulation of mucus and

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inflammatory cells in airway secretions and impaired lung function without increased respiratory effort at rest (Couëtil et al., 2007). The prevalence of IAD in racehorses has been reported to be between 11.2% and 50% (McNamara et al., 1990; Burrell et al., 1996; Wood et al., 2005) and there is evidence that the condition impairs athletic performance (Couëtil et al., 2001; Sanchez et al., 2005) and is an important cause of wastage in the industry (Bailey et al., 1999). Diagnosis is achieved typically by endoscopic examination of the trachea for increased mucopus and cytological examination of secretions obtained from the lower respiratory tract by tracheal wash or bronchoalveolar lavage (BAL) (Moore et al., 1995; Couëtil et al., 2007). Most cases of IAD in horses have increased relative counts of neutrophils in BAL fluid (BALF); less commonly eosinophils and/or mast cells may be increased (Hare and Viel, 1998; Robinson and Hoffman, 2003; Couëtil et al., 2007).

While epidemiological and cytological characteristics of IAD have received considerable research attention, the molecular pathways and role of the immune system in the pathogenesis of IAD remain poorly defined. Another respiratory condition of horses, recurrent airway obstruction (RAO), characterised by pulmonary inflammation, reversible airway obstruction and increased respiratory effort at rest, has a multifactorial aetiopathogenesis that includes a hypersensitivity component and non-specific responses to environmental antigens (Halliwell et al., 1993; McGorum et al., 1993; Pirie et al., 2003; Horohov et al., 2005). Attempts at understanding the immunological basis, including molecular characteristics, of RAO have been made. Using expression of mRNA for various cytokines in BALF, a polarised Th2 cytokine response in horses with RAO has been reported in several studies (Lavoie et al., 2001; Bowles et al., 2002; Cordeau et al., 2004), supporting the involvement of allergy. However, other authors have reported detection of Th1 or mixed Th1/Th2 cytokine responses (Giguère et al., 2002; Ainsworth et al., 2003a; Horohov et al., 2005) or a lack of a Th1 or Th2 cytokine response (Kleiber et al., 2005) in horses with RAO. In addition to Th cytokine responses, increased expression of TNF- α , IL-1 and IL-8 mRNA in horses with RAO has been reported (Franchini et al., 2000; Giguère et al., 2002; Ainsworth et al., 2003a; Laan et al., 2006a), consistent with the induction of an innate immune response and neutrophil recruitment into the airways in this disease. In contrast to RAO, few studies of cytokine mRNA expression in BALF of horses with IAD have been reported. Ryhner et al. (2008) found increased airway mucus in horses with IAD was not associated with alterations in IL-13 or IFN- γ , while in another study, mRNA expression of IL-4 and IL-5 was not increased in 5 horses with transient pulmonary eosinophilia (Riihimäki et al., 2008). Effects of exercise on cytokine expression in equine BALF have been investigated (Ainsworth et al., 2003b; Davis et al., 2005, 2007), however the pattern of cytokine mRNA expression in horses with IAD in comparison to healthy horses is unknown. Elucidating molecular characteristics of IAD, including cytokine responses, may allow a greater understanding of the possible aetiopathological pathway(s) involved and could contribute to development of novel treatments for the disorder.

The aim of the study was to investigate mRNA expression of Th1-type (IL-2, IFN- γ), Th2-type (IL-4, IL-13) and pro-inflammatory cytokines (IL-1 β , IL-17, IL-23, TNF- α) and cytokines involved in neutrophil chemotaxis and activation (IL-8, IL-17) in bronchoalveolar cells in horses with IAD in comparison to age, breed and sex matched horses without evidence of respiratory tract disease.

2. Materials and methods

2.1. Animals

Thirty-two athletic horses referred to the University of Bristol Jim Joel Equine Sports Medicine Centre that underwent tracheal endoscopy and BAL as part of clinical investigation for abnormal respiratory noise and/or poor performance between June 2006 and February 2007 and six ex-racehorses maintained in a research herd of the University of Bristol were included in the study. Horses with historical and/or clinical evidence of RAO (e.g., increased respiratory effort at rest) were excluded from the study. In addition, horses were excluded if there were systemic signs of inflammation/infection (fever, dull demeanour, haematological abnormalities compatible with infection). There were 32 Thoroughbreds, 2 Warmbloods, 1 cob, 1 Arabian, 1 Standardbred and 1 Irish draft cross thoroughbred horse used in the study. There were 27 geldings, 5 entire males and 6 females and the mean ($\pm$ SD) age of the horses was 6.4 ($\pm$ 3.0) years (range 2–15). Athletic horses were used for racing ($n=24$), eventing ($n=4$) dressage ($n=2$), showjumping ($n=1$) or polo ($n=1$). Athletic horses were presented with a complaint of poor performance and an abnormal respiratory noise during exercise ($n=19$), poor performance alone ($n=11$) or abnormal respiratory noise during exercise alone ($n=2$). Horses were allocated to case (IAD) or control groups based on BALF cytology findings. The study complied with the ethics and welfare requirements of the Faculty of Veterinary medicine, University of Glasgow.

2.2. Tracheal endoscopy

Endoscopic examination of the lower airways was performed between 30 and 60 min after a treadmill exercise test to fatigue. Prior to endoscopy, horses were sedated with detomidine hydrochloride (0.01 mg/kg i.v.) and butorphanol tartrate (0.02 mg/kg i.v.). A 1.8 m video endoscope was advanced through the rima glottidis into the trachea as far as the tracheal bifurcation. A visual inspection was carried out to determine the presence of mucus in the trachea and subjectively assess amount of any mucus accumulation. Mucus accumulation was scored using a previously described 0–5 grading system (Gerber et al., 2004).

2.3. Bronchoalveolar lavage

BAL was performed after tracheal endoscopy using a proprietary BAL catheter (Arnolds Ltd., Shrewsbury, UK). A total of 300 ml of sterile pre-warmed 0.9% saline was infused and aspiration was performed manually. Samples were transferred to 10 ml EDTA tubes and sub-

mitted to Langford Veterinary Diagnostic Services for cytological analysis and the remainder of the BALF was transferred to plain 50 ml falcon tubes for further processing. All samples were processed within 1 h of collection.

2.4. Sample processing

The samples of BALF were mixed on a rotational mixer. The cytospin preparations were made by adding two drops of BALF to a cytospin funnel which were then centrifuged at 1500 rpm for 5 min (Cytospin 3 centrifuge, Shandon Scientific Ltd., Astmoor, UK). The prepared slides were stained using a modified Wright's stain. Slides were examined using light microscopy at high power (40 $\times$) to perform a differential cell count of inflammatory cells by counting 300 cells. Horses were assigned to case (IAD) or control groups using the following criteria: case: >5% neutrophils and/or $\geq$ 5% mast cells in BALF; control: <5% neutrophils and <5% mast cells in BALF.

The remaining BALF of each sample was spun for 10 min in a countertop centrifuge at 2000 rpm (at 4 °C). Two 15 ml aliquots of supernatant were retained per sample and the remainder was discarded. The cell pellet was re-suspended in 1 ml sterile PBS (at 4 °C), transferred to one or more screw top Eppendorf tube(s) and spun at 13,000 rpm for 1 min. The remaining supernatant was discarded and the cell pellets and supernatants were stored at –80 °C until transportation to the Division of Infection and Immunity, University of Glasgow. During transportation, samples were maintained frozen in dry ice, and on arrival at the University of Glasgow, the samples were stored at –80 °C until PCR analysis.

2.4.1. RNA extraction

Total RNA was extracted from cell pellets (RNeasy Kit, Qiagen), DNase treated and RNA quality assessed using a Nanodrop spectrophotometer.

2.4.2. Quantitative real-time reverse transcriptase (qRT)-PCR analysis

Quantitative reverse transcriptase PCR (qRT-PCR) assays were performed, in triplicate, using previously described primers and fluorogenic probes for equine cytokines IL-13, IL-17 (Ainsworth et al., 2003a), IL-8, IL-2, IL-1 β , TNF- α (Davis et al., 2005), IFN- γ , and IL-4 (Ainsworth et al., 2003b). Primers and probe specific for equine IL-23 p19 (sequence accession number: AY704416) were designed using Primer Express software (Applied Biosystems). IL-23 primer sequences were eqIL23f (5'- CTG GCC TGG AGT GCA CAT C -3') and eqIL23r (5'- TTG TAG TCT CAG CAT CTC CCT CTT C -3') and probe eqIL-23p (5'- CCA ATG GGA CAT GTG GAT CTA CCA A-BHQ -3'). Assays were also performed to measure expression of β -actin, a house-keeping gene expressed at comparable levels in resting and stimulated or proliferating BALF cells (Ainsworth et al., 2006) and CD-3 ξ expressed on T lymphocytes (Horohov et al., 2005) using reported primers and probes. Standard curves were constructed using serial dilutions of BALF cell mRNA in order to determine amplification efficiency and allow data analysis via the comparative standard curve

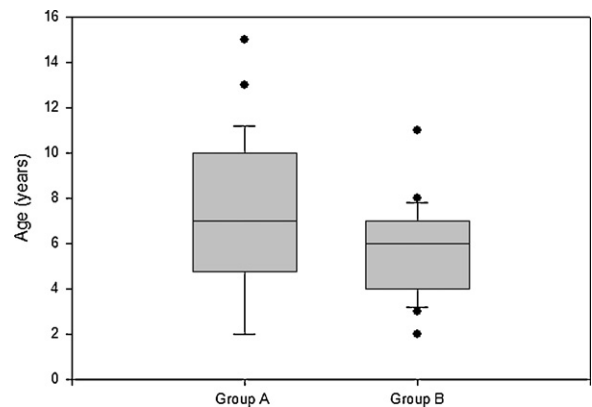


Fig. 1. Age distribution of horses with inflammatory airway disease (group A), compared to control horses (group B). The mean age of horses in group A was 7.0 years and in group B 5.7 years, with no significant difference between groups ($p=0.179$; t test).

method, with normalisation of cytokine expression to β -actin expression.

2.5. Statistical analysis

Relative expression data for each gene of interest, normalised to β -actin expression, was compared between horses with inflammatory airway disease and unaffected controls, using SigmaStat software (Version 3.5, Systat Software). Data was compared between groups using a Mann-Whitney rank sum test or, where normally distributed following log transformation after the addition of a constant of 1, by an unpaired t test. Distribution of age and gender between groups was compared using a t test and Chi square test respectively. A Fisher's exact test was performed to compare tracheal mucus scores between groups. For all analyses, differences between groups were considered statistically significant where $p < 0.05$.

3. Results

3.1. Horse disease status classification

On the basis of BALF cytology, 21 horses were diagnosed with IAD and 17 were assigned as controls. For the IAD group, there were 13 geldings, 3 entire males and 5 mares. Mean age of the IAD group was 7.0 ± 3.6 years (range; 2–15). There were 14 geldings, 2 entire males and 1 female in the control group, which had a mean age of 5.7 ± 2.1 years (range; 2–11). There was no significant difference in mean horse age between groups ($p=0.18$) (Fig. 1). There was no significant difference in gender distribution between groups ($p=0.515$; Chi square test).

3.2. Tracheal endoscopy

The median tracheal mucus scores for horses with IAD and control horses were both 1 using the 0–5 grading scale described by Gerber et al. (2004). For both groups the range in scores was 0–3. There was no significant difference between groups ($p=0.57$).

Table 1

Differential cell counts of inflammatory cells in BALF for horses with IAD (cases) and control horses. For horses with IAD, differential cell counts that are above the pre-determined cut-off values for diagnosis of IAD are presented in bold.

Neutrophils (%)	Mast cells (%)	Eosinophils (%)	Macrophages (%)	Lymphocytes (%)
Cases				
17.2	4.0	0.0	55.6	23.2
8.0	0.0	0.0	21.0	71.0
0.0	10.0	0.0	88.0	2.0
16.0	0.0	0.0	74.0	10.0
4.1	5.2	0.0	73.2	17.5
6.2	3.1	0.0	64.9	25.8
6.0	3.0	0.0	56.0	35.0
24.0	0.0	0.0	76.0	0.0
15.3	0.0	0.0	49.0	35.7
15.0	0.0	0.0	81.0	4.0
6.0	16.0	0.0	70.0	8.0
0.0	5.0	0.0	93.0	2.0
2.0	5.0	1.0	50.0	42.0
2.0	8.0	1.0	69.0	20.0
7.1	4.0	0.0	59.6	29.3
10.8	4.3	0.0	66.7	18.3
3.0	5.0	0.0	56.0	36.0
14.1	2.2	0.0	67.4	16.3
6.3	3.8	0.0	67.5	22.5
5.1	2.0	0.0	58.5	34.3
9.8	5.4	0.0	60.7	24.2
Controls				
4.8	0.0	0.0	78.5	16.7
2.1	0.0	0.0	74.9	23.0
4.8	3.8	0.0	64.3	27.0
4.0	4.0	0.0	74.0	18.0
1.0	1.0	0.0	60.0	38.0
3.3	0.0	0.0	78.0	18.7
3.0	0.0	3.0	59.0	35.0
2.1	4.2	0.0	82.1	11.6
2.0	4.0	1.0	73.0	20.0
5.0	0.0	3.0	72.0	20.0
1.0	0.0	0.0	86.5	12.5
2.0	3.0	0.0	68.0	27.0
4.0	3.0	0.0	62.6	30.3
4.0	2.0	0.0	78.0	16.0
3.0	0.0	0.0	22.0	75.0
4.2	0.0	0.0	84.4	11.5
5.0	1.0	0.0	43.0	51.0

3.3. Bronchoalveolar lavage

Results of cytological examination of BALF from the 38 horses are provided in Table 1. Twenty-one horses had cytological findings of IAD using the pre-determined inclusion criteria for the study; 15 horses had increased percentage of neutrophils (5.1–24.0%) and eight horses had increased percentage of mast cells (5.0–16%). Two horses had increased percentages of both neutrophils and mast cells.

3.4. Upregulation of cytokine mRNA expression in horses with IAD

Analysis of mRNA expression, normalised to β -actin, in BALF cells revealed a significantly greater expression of IL-1 β ($p=0.046$), IL-23 (p19 subunit) ($p=0.027$), and TNF- α ($p=0.014$) mRNA in horses with IAD than the control group (Fig. 2). No significant difference between groups was detected for the mRNA of cytokines characteristic of a Th1-type (IL-2, IFN- γ) or Th2-type (IL-4, IL-13) immune response ($p>0.05$; Mann-Whitney rank sum test) (Fig. 2).

There was no significant difference in the expression of CD-3 ξ between IAD and control groups ($p>0.05$; Mann-Whitney rank sum test).

4. Discussion

The results of the current study indicate that in the population of horses examined there was no evidence of a polarised T-cell response in horses with IAD. The increased expression of mRNA of the pro-inflammatory cytokines IL-1 β , TNF- α and IL-23 in horses with IAD suggests that airway neutrophilia and/or increased mast cells are associated with active cell recruitment and an inflammatory process in the distal airways. The increased cytokine expression in the horses with IAD is unlikely to represent an immune response to a specific antigen and may result from exposure to varied antigens (non-infectious and infectious). Further, the inflammatory process is likely to involve numerous cells types, including alveolar macrophages, lymphocytes and respiratory epithelial cells, as has been found in horses with RAO (Cordeau et al., 2004; Ainsworth et al., 2006; Laan et al., 2006a). Given the lack of a significant difference in the

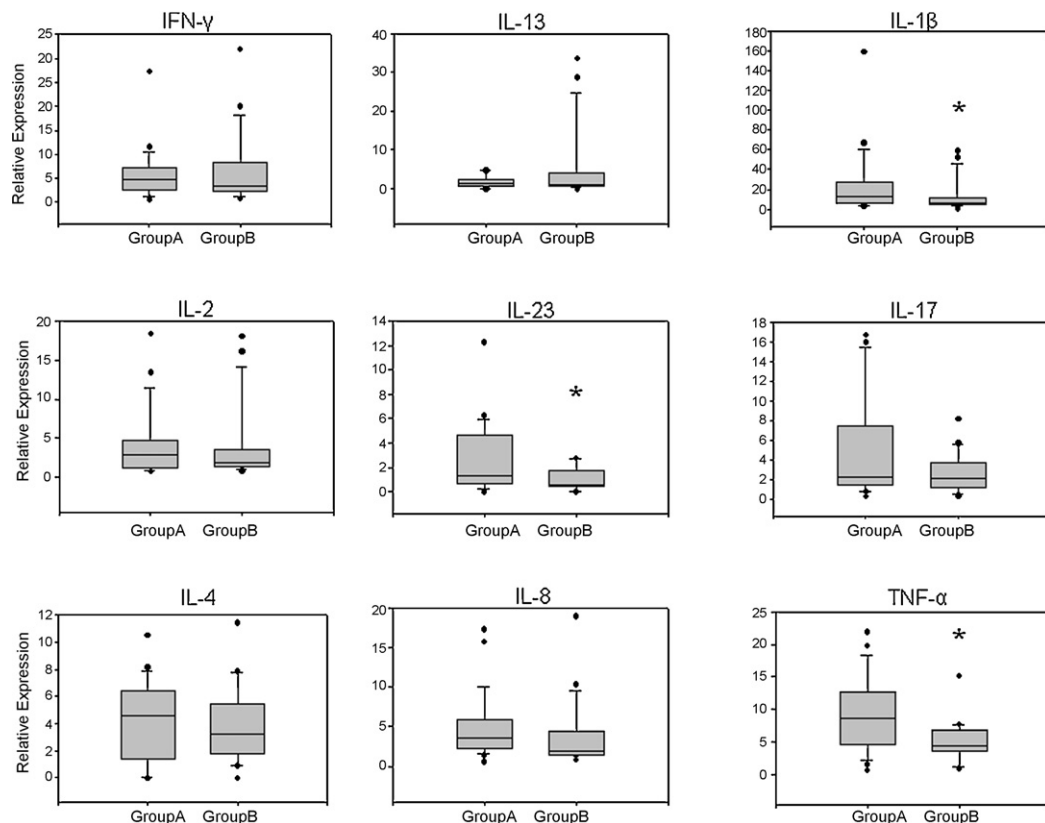


Fig. 2. Relative expression of IFN- γ , IL-13, IL-1 β , IL-2, IL-23, IL-17, IL-4, IL-8 and TNF- α mRNA, normalised to β -actin mRNA, in horses with inflammatory airway disease (group A), compared to control horses (group B). The relative expression of IL-1 β , IL-23 and TNF- α were significantly different between groups ($p < 0.05$). The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles and individual outliers are shown.

expression of CD-3 ξ between IAD and control groups, it is unlikely that total T lymphocyte numbers differ between control and IAD horses, such that this would not contribute to the observed differences.

Interleukin-1 β and TNF- α are pro-inflammatory cytokines that may be important components of pulmonary innate immune responses in horses, and may be involved in the pathogenesis of IAD as suggested by the results of our study of naturally-acquired disease. Similarly, evidence of increased production of IL-1 β and/or TNF- α has been found in several experimental studies of horses with RAO, supporting a role of these cytokines in lower airway diseases. Exacerbation of clinical disease in RAO-susceptible horses by exposure to mouldy hay resulted in increased mRNA expression of BALF IL-1 β and TNF- α in a study by Giguère et al. (2002). Laan et al. (2006a) found increased expression of mRNA of these cytokines in isolated alveolar macrophages from RAO horses compared to healthy horses after inhalational challenges with LPS, hay dust suspension (HDS) or *Aspergillus fumigatus* extract (AF), suggesting differences in innate immune responses exist between healthy horses and those susceptible to RAO. Further, Laan et al. (2005) reported an increased production of IL-1 β and TNF- α protein by alveolar macrophages after exposure to HDS

or AF. Conversely, Ainsworth et al. (2007) found in vitro exposure to hay dust did not result in increased expression of IL-1 β mRNA by pulmonary mononuclear cells isolated from horses with RAO compared to control horses. In other studies, treatment of RAO-affected horses with clenbuterol or fluticasone propionate has been shown to result in reduced expression of IL-1 β and/or TNF- α mRNA in BALF (Laan et al., 2006b; DeLuca et al., 2008), consistent with a role of these cytokines in airway inflammation. Increased expression of IL-1 β and TNF- α mRNA in BALF has been found also in horses that have been exercised while breathing cold air (Davis et al., 2007), suggesting up-regulation of inflammatory pathways in the lower airways exposed to unconditioned air. The aetiopathogenesis of IAD remains poorly understood, however it is possible the increased expression of IL-1 β and TNF- α in horses with IAD in the current study is reflective of airway exposure to various aerosolised particles, including LPS, vegetative material, micro-organisms and fungal antigens, noxious gases or unconditioned air (Couëtil et al., 2007). While LPS challenge studies in horses with IAD have not been reported, Malikides (2003) found a positive association between environmental LPS exposure and development of neutrophilic IAD in racehorses. Similarly, LPS has been shown to have synergistic effects with organic dust on

development of airway neutrophilia in heaves-susceptible horses (Pirie et al., 2003).

Interleukin 23, a member of the IL-12 family, has important roles in innate and acquired immune responses, including host defence against bacterial infection (Iwakura and Ishigame, 2006). However, IL-23 is also considered to be involved in the pathogenesis of chronic inflammatory diseases, including asthma, arthritis and inflammatory bowel diseases in humans (Iwakura and Ishigame, 2006; Nakajima and Hirose, 2010). Horses with IAD in our study had an increased expression of IL-23 mRNA which may indicate a role in the development of lower airway inflammation. Observations of cytokine responses in murine models of inflammatory diseases have revealed antigen-stimulated macrophages produce IL-23, which promotes development of Th17 cells (IL-23–Th17 axis) and may also act on macrophages in an autocrine/paracrine manner to stimulate the generation of pro-inflammatory cytokines, including IL-1 β and TNF- α (Iwakura and Ishigame, 2006). While there was no increased expression of IL-17 (product of Th17 cells) in horses with IAD in our study, the increased expression of IL-23, IL-1 β and TNF- α in these horses may be reflective of macrophage involvement in antigenic responses in the lower airways of affected horses.

In the current study, there was no evidence of a polarised T cell response in horses with IAD. Conversely, several studies have demonstrated cytokine profiles consistent with T cell responses in horses with RAO. Evidence of a polarised Th2 cytokine response in horses with RAO has been reported (Lavoie et al., 2001; Bowles et al., 2002; Cordeau et al., 2004), supporting previous evidence of a role of allergy in the disease (Halliwell et al., 1993; McGorum et al., 1993). However, other authors have found Th1 or mixed Th1/Th2 cytokine responses (Ainsworth et al., 2003a; Giguère et al., 2002; Horohov et al., 2005) or a lack of a Th1 or Th2 cytokine response (Kleiber et al., 2005) in horses with RAO. Differences between these studies may reflect variations in disease phenotype or study protocol or time-dependant expression of specific cytokines. Given the heterogeneity of the cytological findings and airway dysfunction in IAD (Couëtil et al., 2007), it remains possible that some horses with IAD have a polarised T-cell response in the lower airways and that we were unable to detect any significant differences in mRNA expression of Th1 or Th2 cytokines due to our small group numbers. Continued research of the molecular characteristics of horses with IAD is required to inform our understanding of the pathogenesis of the syndrome.

In the current study, horses with IAD were defined by increased relative percentages of neutrophils (>5%) and/or mast cells (>5%) in BALF. The described alterations in cellular analysis of BALF from horses with IAD are diverse and include increased total nucleated cell, neutrophil, lymphocyte and macrophage counts and increases in relative percentages of neutrophils, eosinophils or mast cells (Moore et al., 1995; Hare and Viel, 1998; Couëtil et al., 2007). Recently, cytological criteria for IAD have been proposed in an attempt to define the syndrome and include >5% neutrophils, >2% mast cells and/or >0.1% eosinophils (Robinson and Hoffman, 2003; Couëtil et al., 2007). Using this definition, several of our control horses would be con-

sidered to have mast cell-associated IAD and be at risk for airway hyper-reactivity as described by Hoffman et al. (1998). However, several studies have documented BALF mast cell percentages >2 and <5% in apparently healthy horses (Sweeney et al., 1992, 1994; McGorum and Dixon, 1994; Holcombe et al., 2001; Gerber et al., 2003), suggesting percentages >2% may be a normal finding. Variation in mast cell percentages in BALF between studies may reflect differences in environment, climatic conditions and horse characteristics. As a result of these studies, and our clinical impression that horses without respiratory disease in the UK have up to 5% mast cells in BALF (unpublished data), we selected a mast cell cut-off value of 5% for the current study.

Eosinophils are an uncommon finding in BALF (McGorum and Dixon, 1994; Hughes et al., 2003), which was the situation also in the current study where only four horses had eosinophils detected cytologically. While increased relative percentages of eosinophils in equine BALF have been associated with pulmonary hyper-responsiveness (>6%) (Hare and Viel, 1998) and IAD (>0.1–3%) (Hare et al., 1994; Couëtil, 2002; Couëtil et al., 2007), increases may be transient and of uncertain clinical importance (Gerber et al., 2003). In the current study, horses with eosinophils in BALF had percentages $\leq$ 3%, in the absence of elevated mast cells and neutrophils. These animals were not considered to have IAD as the clinical relevance such low percentages of eosinophils is uncertain, while other authors have designated a criteria of an eosinophil percentage of >3% as significant in pulmonary disease (McGorum and Dixon, 1994; Couëtil, 2002).

Thirty-two of the 38 horses in our study had BALF collected after strenuous exercise on a high-speed treadmill, and it is possible mucosal responses to exercise influenced airway cytology and/or cytokine mRNA expression. Exercise in racehorses has been shown to have no effect on relative percentages of leukocytes in BALF (Clark et al., 1995) and as such, it is unlikely exercise influenced the cytological results in our study. Exposure of the intrapulmonary airways to cold air (5 °C) compared to warm air (25 °C) during exercise of healthy horses has been shown to cause a preferential increase in expression of mRNA of Th2 cytokines (IL-4, IL-5, IL-10) at 5 h after exercise (Davis et al., 2005) followed by a shift to a profile more characteristic of generalised inflammation (IL-1 α , IL-1 β , IL-6, IL-8, TNF- α) at 24 and 48 h after exercise (Davis et al., 2007). In the current study, while the temperature of inspired air was not recorded, most horses were examined during the summer months (data not shown) and inspired air during exercise was likely in the region of 10–20 °C. In addition, BALF was collected from horses within 2 h of treadmill exercise (similar to the 5 h time-point of Davis et al. (2005)) and no Th2 cytokine response was detected. In an earlier study, mRNA expression of IL-4, IL-12 and IFN- γ in BALF of healthy horses was unaffected by strenuous exercise (Ainsworth et al., 2003a,b), suggesting the exercise regimen used in our study was unlikely to have influenced the results. However, the studies of airway cytokine responses to exercise in horses used healthy animals and it is unknown whether different responses develop in horses with pre-existing airway inflammation.

Inflammatory airway disease is likely a heterogeneous syndrome, given the variability in described cytological profiles and spectrum of possible aetiological agents (e.g., inorganic/organic particles, micro-organisms, noxious gases, unconditioned air), which may be involved in development of disease (Couëtil et al., 2007). In the current study, we examined horses with IAD collectively as one group and did not account for heterogeneity in cytological findings. However, it is possible that different cytological profiles in horses with IAD may reflect underlying differences in inflammatory pathways and thus have different cytokine mRNA expression profiles. Recently, BALF cytology of horses with IAD was reported to be associated with specific clinical responses; horses with airway neutrophilia were more likely to cough, while airway hyper-responsiveness was found in horses with mast cell associated IAD (Bedenice et al., 2008). The findings of that study provide support to the possibility of IAD-type dependant biological responses, and as such, investigations of the molecular profiles of specific IAD types (e.g., neutrophilic, eosinophilic and mast cell-associated IAD) is warranted in future studies. Clearly, given the heterogeneity evident in our study population this would require the recruitment of a large number of animals. An association between IAD (as defined by tracheal inflammation) and bacterial infection in young racehorses has been described previously (Burrell et al., 1996; Wood et al., 2005). In the current study we did not investigate for bacterial infection of the lower airways and bacterial involvement in horses with IAD cannot be excluded. However, we considered this unlikely as bacteria have not been associated with IAD diagnosed using BALF cytology (Couëtil et al., 2007) and the prevalence of bacterial isolation from the lower airways in racehorses decreases dramatically from 2 to 4 years of age (Burrell et al., 1996; Wood et al., 2005), whereas the mean age of horses in the current study was 6.4 years. Additionally, no extracellular or intracellular bacteria were observed during cytological examination of BALF samples in our study (data not shown).

An important limitation of the current study is the assumption that mRNA expression levels recorded were reflective of the concentrations of the biologically active cytokine. It remains possible that increased expression of mRNA is not an accurate marker for protein expression due to alterations at the level of mRNA transcription or post-transcription regulation. Direct measurement of cytokine protein concentrations would be desirable to confirm cytokine expression patterns of horses with IAD, however were not performed in the current study. Perkins et al. (2008) found that both mRNA expression and protein concentrations of IL-8 and IFN- γ in BALF of healthy horses were unaffected by histamine bronchoprovocation, suggesting mRNA expression may be a valid proxy for cytokine protein expression. However, in the same study, mRNA expression for IL-4 was detected, while IL-4 protein was not detected at any time point (Perkins et al., 2008). It is unknown whether this finding was due to methodological issues or discordance between mRNA and protein expression.

In conclusion, the results of this study indicate that IAD of horses is associated with increased mRNA expres-

sion of pro-inflammatory cytokines in the cells collected by BAL from lower airways, which may reflect stimulation of the innate immune responses to various inhaled antigens. There was no evidence of a polarised T-cell cytokine response in horses with IAD, which suggest hypersensitivity responses may not be involved in the aetiopathogenesis of IAD. However, studies of the temporal regulation of cytokine mRNA expression and quantification of cytokine protein in the lower airways are required to better characterise the molecular responses in horses with IAD and obtain greater understanding of the aetiopathogenesis of this condition.

Conflicts of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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