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Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction

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Abstract

Acute and chronic inflammation of the airway remains an important health problem for equids. “Heaves” or recurrent airway obstruction (RAO) remains one of the most commonly diagnosed conditions affecting the lung of older horses in Europe and the United States. The typical clinical signs of RAO include non-productive coughing, serous nasal discharge, labored expiratory effort, and flaring of the nostrils. Auscultation of the lungs of the affected horse often reveals abnormal respiratory sounds, described as crackles and wheezes, throughout the area of the lung field. These clinical signs occur secondary to an inflammatory response that results in bronchospasm, excessive mucus production and airway obstruction. This inflammatory response is characterized by the presence of excessive mucus and inflammatory cells, primarily neutrophils, in the small airways. Most evidence suggests that RAO is the result of a pulmonary hypersensitivity to inhaled antigens. Exposure of affected horses to hay dust, pollens, and mold spores leads to neutrophil accumulation in the lung and bronchospasm. The identification of allergen-specific IgE in bronchoalveolar lavage (BAL) fluid and sera of affected horses supports the involvement of a late phase, IgE-mediated, hypersensitivity reaction in the pathogenesis of equine RAO. The production of IgE antibodies is regulated by the cytokines IL-4 and IL-13. Using a quantitative PCR method we have reported that horses with RAO exhibit a modified Type 2 cytokine response characterized by the production of IL-4 and IL-13 mRNA, but not IL-5 mRNA in BAL cells. Interferon-gamma mRNA was also elevated, suggesting a mixed response. While these results are consistent with equine RAO being the result of an aberrant Type 2 cytokine response to inhaled allergens, others have failed to find any evidence of elevated Type 2 cytokine mRNA in BAL from horses with “heaves”. It is likely that these disparate results could be the result of differences in the clinical stage of the affected animals or the timing of sample collection. Here, we report a diverse pattern of cytokine gene expression when sampling a group of affected horses over a period of time.

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1. Introduction

“Heaves” or chronic obstructive pulmonary disease (COPD) or, more recently, recurrent airway obstruction (RAO) remains one of the most commonly

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diagnosed conditions affecting the lungs of older horses in Europe and the northern United States (Bracher et al., 1991; Larson and Busch, 1985; McPherson et al., 1978). A clinically similar condition has been reported in horses on pasture during the summer months and is referred to as summer pasture-associated obstructive pulmonary disease (SPAOPD) (Costa et al., 2000; Dixon et al., 1995a; Mair, 1996; Seahorn et al., 1996). Though fewer than 10% of COPD-affected horses also suffer from SPAOPD (Dixon et al., 1995a), clinical signs and cellular composition of tracheal and bronchoalveolar lavage fluid suggest a common pathogenic mechanism (Costa et al., 2000; Mair, 1996; Seahorn et al., 1996).

The typical clinical signs of RAO include non-productive coughing, serous nasal discharge, labored expiratory effort, and flaring of the nostrils (Dixon et al., 1982, 1995b; Seahorn et al., 1996). Auscultation of the lungs of the affected horse often reveals abnormal respiratory sounds, described as crackles and wheezes, throughout the area of the lung field. These clinical signs occur secondary to an inflammatory response that results in bronchospasm, excessive mucus production and airway obstruction. This inflammatory response is characterized mainly by the presence of neutrophils in the small bronchioles and the release of inflammatory mediators including histamine, serotonin, leukotrienes, thromboxane, and 15 HETE (Franchini et al., 1998; Gray et al., 1992; McGorum et al., 1993a). These inflammatory mediators are responsible for the bronchospasm, mucus secretion, airway hyperreactivity, and airway wall thickening, which create airway obstruction (Bayly and Slocombe, 1997; Dixon, 1992; Kaup et al., 1990c). With each bout of inflammation, further anatomical changes occur within the airway wall such as proliferation of smooth muscle, goblet cell metaplasia, and thickening of the mucosa (Kaup et al., 1990c; Robinson and Derksen, 1998).

Though dietary toxicity, antiprotease deficiency, bacterial-derived proteases, and viral respiratory infection have been proposed as possible causes of RAO in horses, most evidence suggests that it is the result of a pulmonary hypersensitivity to inhaled antigens (Eyre, 1972; McGorum et al., 1993b; Thomson and McPherson, 1983). Exposure of affected horses to hay dust, pollens, and mold spores leads to neutrophil accumulation in the lung and bronchospasm

(Marr et al., 1996; McGorum et al., 1993b). Histamine concentrations in bronchoalveolar lavage fluid are significantly correlated with the numbers of metachromatically staining cells, presumed to be mast cells and/or basophils (McGorum et al., 1993a). Basophil degranulation in vitro in response to stimulation with putative allergens (Halliwell et al., 1993) and allergen-specific IgE in BAL fluid and sera of affected horses (Schmallenbach et al., 1998) support the involvement of a late phase, IgE-mediated, hypersensitivity reaction in the pathogenesis of equine RAO. Interestingly, there is little evidence of an immediate hypersensitivity response in allergen-challenged horses.

The production of IgE antibodies is regulated by the cytokines interleukin (IL)-4 and IL-13 (Wills-Karp et al., 1998; Yssel et al., 1998). Two laboratories using a quantitative PCR method have reported that bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMC) of RAO-affected horses expressed IL-4 mRNA but not IL-5 in BAL cells (Beadle et al., 2002; Giguere et al., 2002). Interferon-gamma (IFN- γ) mRNA was also elevated in both studies. By contrast, Lavoie et al. reported a classical Type 2 cytokine response including both IL-4 and IL-5 mRNA production in horses affected with "heaves" (Cordeau et al., 2004; Lavoie et al., 2001). Interleukin-5 plays a central role in the eosinophilic inflammation characteristic of certain forms of human and murine asthma (Wills-Karp, 1999). The most notable feature of equine RAO is the predominant neutrophilia in the airways (Dixon et al., 1995c; Franchini et al., 1998; McGorum and Dixon, 1993). This bronchiolar neutrophilia may be the consequence of the over-expression of cytokines chemotactic for neutrophils, particularly IL-8 (Franchini et al., 1998; John et al., 1998). Higher amounts of IL-8 and increased numbers of neutrophils are found in horses with COPD (Franchini et al., 1998) and thus serves as a marker of this disease. While increased expression of IL-8 can account for the increased numbers of neutrophils recruited to the airway, their persistence in the airway is probably related to other mechanisms. The finding that NF- κ B activity is aberrantly sustained in the bronchial cells of heaves-affected horses after allergen elicitation suggests that this could account for the continued presence of inflammatory cells in the affected airway (Bureau et al., 2000). It seems likely

that this increase in NF- κ B activity is the result of the presence of Type 2 cytokines that can regulate its production and turnover (Lin et al., 2000). While these results are consistent with equine RAO being the result of an aberrant Type 2 cytokine response to inhaled allergens, a fourth group, also using a PCR approach, failed to find any evidence of elevated Type 2 cytokine mRNA in BAL from horses with “heaves” and instead noted elevated amounts of INF- γ and IL-8 mRNA (Ainsworth et al., 2003). These disparate results could be due to the different methodologies employed to measure cytokine-specific mRNA. It is perhaps more likely that difference in the clinical stage of the affected animals or the timing of sample collection contributed to these discrepancies. Here, we have addressed some of these possibilities by following a group of horses affected with SPAOPD throughout the season and assessed their BAL and PBMC for cytokine mRNA production.

2. Materials and methods

2.1. Horses

Six mixed breed horses previously diagnosed with the SPAOPD form of equine RAO and six mixed breed controls were used in this study. All horses were vaccinated once a year for Eastern, Western, and Venezuelan encephalitis, tetanus, and equine influenza virus. All horses were treated with anthelmintics on a recommended schedule. The RAO-affected horses and controls were kept on the same pasture throughout the year and sampled prior to the appearance of clinical signs (June), during the time when they were symptomatic (July, August), and later in the year when asymptomatic (October, January, March). All horses had rectal temperature, heart rate, respiratory rate, nostril flare, and abdominal lift recorded daily. A clinical score (CS) was calculated using a score assigned to nostril flare and abdominal lift associated with respiration (Seahorn et al., 1997). Affected horses with clinical scores >2.5 were selected for sampling along with a control horse.

At sampling, the RAO-affected horses along with their control horse were brought into a barn for pulmonary function testing and bronchoalveolar lavage sample collection. Blood was also collected via

jugular venipuncture. The horses were returned to pasture the next day if clinical signs permitted. Those horses exhibiting severe signs were kept in the barn until their symptoms regressed. Horses were treated as necessary for secondary bacterial infections and for worsening clinical signs. All procedures were approved by Louisiana State University’s Institutional Animal Care and Use Committee.

2.1.1. Bronchoalveolar lavage

Horses were sedated using xylazine hydrochloride (0.6 mg/kg, i.v.) and bronchodilated using glycopyrrolate (0.0022 mg/kg, i.v.). Following placement of a nose twitch, a 218-cm (8-mm diameter) flexible fiberoptic endoscope was passed through the nasal passage into the trachea and wedged in the distal airway. A sterile polyurethane tube was inserted through the biopsy port of the endoscope and advanced into the airway. Five 60-ml aliquots of sterile phosphate buffered saline containing 0.2% EDTA were infused by hand with 60-ml syringes (Sweeny and Beech, 1991). Immediately following infusion, bronchoalveolar lavage fluid (BALF) was collected into the same syringe and transferred to a sterile flask. The color and volume of fluid retrieved was recorded for each horse. A portion of the pooled aspirate was set aside for cytologic evaluation. Air-dried smears were stained with a Modified Wright solution and 200 cells classified under high magnification (100 \times) as neutrophils, lymphocytes, alveolar macrophages, mast cells, eosinophils, or epithelial cells, and expressed as percentages of the total count.

2.1.2. Lymphocyte preparations

Lymphocytes were obtained from BAL fluids by initially filtering the fluid through gauze to remove large flecks of mucus. The resulting cell suspension was pelleted, washed once with PBS and counted using trypan blue exclusion dye to assess viability. Heparinized blood samples were collected aseptically from the jugular vein. The blood was centrifuged to obtain a buffy coat and, after dilution with calcium and magnesium free phosphate buffered saline, under-layered with Ficoll–Paque (Pharmacia) and centrifuged at 800 \times g for 30 min (Swiderski et al., 1999b). The PBMC were subsequently extracted from the interface layer, washed in PBS, and counted. The PBMC were fractionated into CD4⁺ and CD8⁺ subsets

using equine-specific monoclonal antibodies and a magnetic bead separation system. The PBMC were labeled with monoclonal antibodies recognizing either the EqCD4 (antibody: CVS4) or EqCD8 (antibody: CVS8) antigens, kindly provided by P. Lunn (Wisconsin). Antibody-labeled cells were then incubated with MACS™ goat anti-mouse immunoglobulin magnetic MicroBeads (MiniMACS, Miltenyi Biotec, Inc., Auburn, CA). After incubation the cells were washed and applied to a separation column placed in a high gradient magnetic field (MiniMACS Separation Column). The column was washed to remove unbound negatively selected cells (depleted cell population), and then removed from the magnetic field to release the antibody-labeled positively selected cells (enriched cell population). Subsequent FACS analysis revealed that PBMC enriched for EqCD8⁺ cells using this method resulted in a purity of 90%, and when PBMC were enriched for EqCD4⁺ cells the resulting population was 97% pure.

2.1.3. *Quantitative (Q)PCR analysis of cytokine production*

For QPCR analysis of cytokine production, 3×10^6 BAL cells or PBMC were placed into an acid guanidium thiocyanate–phenol disassociation buffer (RNA Stat-60, Tel Test) and frozen. Frozen samples were quickly thawed and the RNA extracted using a phenol:chloroform procedure (Chomczynski and Sacchi, 1987). In all cases, OD_{260/280} ratios were greater than 1.9 and RNA yields were greater than 50 µg/ml. One microgram of RNA was reverse transcribed into cDNA in an 80 µl reaction containing 20 units of AMV reverse transcriptase, 0.5 µg of oligo dT primers, 40 units of RNasin, and 5 mM MgCl₂ (Promega, Madison, WI). Primers based on the sequences for equine cytokines (Swiderski et al., 1998, 1999a,b) and CD3-ζ were used to amplify the corresponding cDNAs (Beadle et al., 2002). The cDNA was then amplified and quantitated by “real-time” PCR (ABI Systems 7500 Real-Time PCR Instrument, Foster City, CA) using the Taq thermostable DNA polymerase. For absolute quantitation, a serially diluted plasmids encoding the respective cytokines and CD3-ζ were used to generate standard curves for each amplification. Differences in RNA isolation and cDNA construction between samples were corrected using CD3-ζ mRNA as a housekeeping gene and to adjust for the relative T

cell composition for each sample as this may vary within the BAL sample depending upon the number of neutrophils recruited into the lung during acute disease episodes. Since CD3-ζ is found primarily on T lymphocyte its relative abundance is indicative of the presence of these cells in a mixed population, such as BAL cells. Here, we have adjusted the calculated copy number for each cytokine based on the amount of CD3-ζ mRNA in each sample to adjust for variations in T cell numbers in the BAL of affected versus control horses. Final results are thus reported as corrected copy number normalized to 10,000 copies of CD3-ζ mRNA for each sample.

2.2. *Statistical analyses*

Since the data were not normally distributed, non-parametric tests were used to analyze the results for statistical significance. A repeated measures analysis of variance on ranks was used to detect temporal effects and differences between affected and control horses. Statistical significance was set at $p < 0.05$.

3. Results

3.1. *Clinical signs*

The clinical scores of the SPAOPD-affected horses all exceeded 3.5 during the months of July and August. Clinical signs included flaring nostrils, abdominal lift, and increased mucus in the airway. Clinical signs abated but remained above the 2.5 thresholds in the fall (October). By winter (January, March) clinical signs were absent.

3.2. *BAL composition*

The BAL fluid collected from the affected horses in the summer contained predominantly neutrophils (>60%), as previously reported (Beadle et al., 2002; Costa et al., 2000). There were significantly fewer neutrophils in the airways of the controls (<3%; $p < 0.05$). These profiles persisted throughout the collection periods from July and into October. By January, the percentage of neutrophils (>3.5%) in the airways of the RAO horses was not significantly different from that of the controls.

3.3. Cytokine mRNA production in BAL samples

BAL samples were collected from RAO-affected and control horses throughout the season and cell pellets collected from these samples assayed for cytokine mRNA expression (Fig. 1). While the cytokine mRNA profile of the affected and control animals were indistinguishable at the first sampling point, there was a marked increase in cytokine mRNA concentrations with the advent of clinical symptoms. In particular, IL-13 mRNA in the BAL cells increased throughout the sampling period before decreasing in January. A similar increase was observed in IFN- γ mRNA. By contrast, IL-4 mRNA did not achieve statistical significance until the January sample when IL-13 and IFN- γ mRNA had decreased. There was little IL-5 mRNA in the BAL cells and this did not change with time (data not shown).

3.4. Cytokine mRNA production in PBMC samples

Analysis of mRNA collected from CD4⁺ PBMC throughout the summer revealed a similar pattern to that observed in the BAL cells (Fig. 2). While there was increased IL-13 and IFN- γ mRNA in CD4⁺ cells collected from RAO-affected horses in August, IL-4 mRNA was significantly elevated in January. Once again, no IL-5 mRNA was detected above a background level. No consistent pattern of cytokine gene expression was evident in the CD8⁺ PBMC samples from either the control or RAO-affected horses.

4. Discussion

Prior work on cytokine production in RAO-affected horses has yielded variable patterns of gene expression in the BAL and PBMC (Ainsworth et al., 2003; Beadle et al., 2002; Cordeau et al., 2004; Giguere et al., 2002; Lavoie et al., 2001). While different methodologies employed in these studies could account for some of these disparities, differences in the stage of disease and the timing of sample collection could also be responsible. Here, we show that affected horses exhibit varying amounts of Types 1 and 2 cytokine production throughout the year. Thus, we observed initially increasing amounts of IL-13 and IFN- γ mRNA in

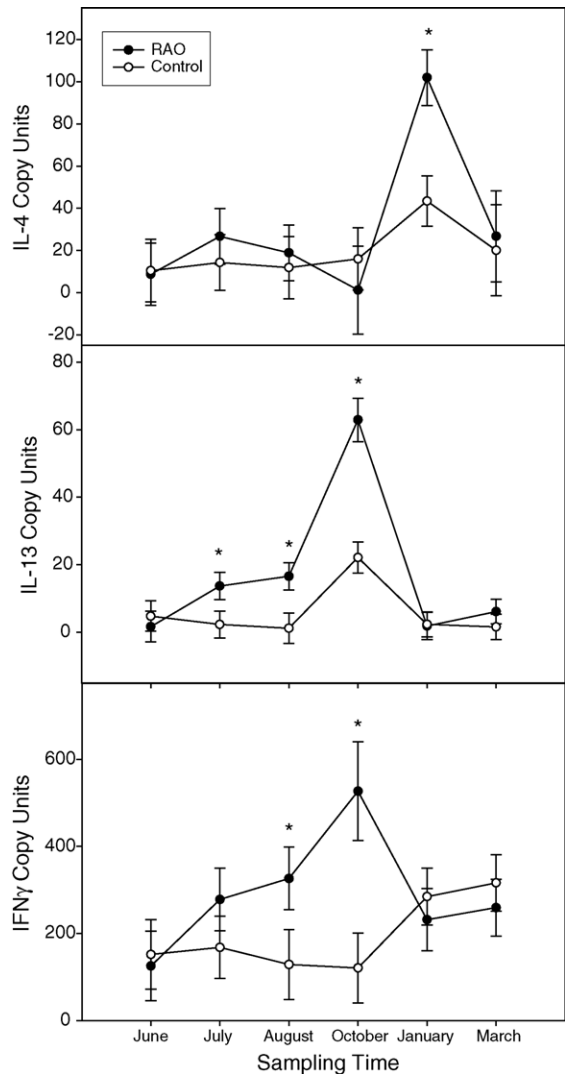


Fig. 1. Cytokine mRNA in BAL of RAO-affected and control horses. BAL samples were collected at the months indicated. Total RNA was isolated from the cell pellets and converted to cDNA using reverse transcriptase and oligo dT primers. Cytokine-specific primers were then used to amplify the corresponding cDNAs. The PCR results were normalized for total T cell components using CD3- ζ mRNA. Each point represents the average \pm S.E. of six control (open circles) and six RAO-affected (filled circles) horses. The asterisk denotes a significant difference between control and RAO horses at $p < 0.05$, RMANOVA.

the BAL and CD4⁺ PBMC of affected horses throughout the summer months. While IL-4 mRNA was also significantly elevated compared to the controls, this only occurred in the winter time when

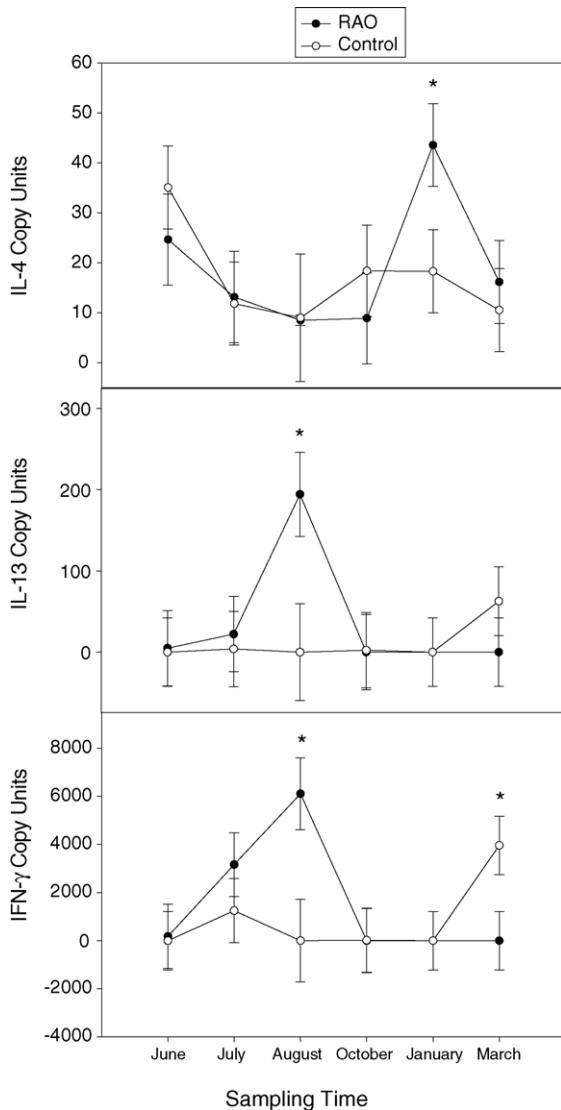


Fig. 2. Cytokine mRNA in the CD4⁺ PBMC collected from RAO-affected and control horses. PBMC samples were collected at the months indicated and the cells fractionated into a CD4⁺ fraction using magnetic bead separation. Total RNA was isolated from the cell pellets and converted to cDNA using reverse transcriptase and oligo dT primers. Cytokine-specific primers were then used to amplify the corresponding cDNAs. Samples were normalized for total T cell components using CD3- ζ mRNA. Each point represents the average \pm S.E. of six control (open circles) and six RAO-affected (filled circles) horses. The asterisk denotes a significant difference between control and RAO horses at $p < 0.05$, RMA-NOVA.

IFN- γ mRNA was also lower. The observation that affected horses had elevated IL-13 mRNA in both their BAL and PBMC is novel but not surprising given the central role this cytokine plays in regulating IgE production (Wills-Karp et al., 1998). These results are consistent with other reports indicating that allergen-specific IgE antibodies play a central role in equine RAO (Halliwell et al., 1993; Schmallenbach et al., 1998). The source of the cytokine production in the blood was CD4⁺ lymphocytes with the CD8⁺ cells exhibiting no definite pattern of cytokine expression amongst either the affected or control horses. The cellular source of the cytokine mRNA in the BAL was not determined and multiple cell types including basophils, mast cells, and eosinophils can produce Type 2 cytokines (Burd et al., 1995; Kuna and Kaplan, 1996; Ochensberger et al., 1996), however it appears likely that the cytokine source in this study were the lymphocytes since analysis of BAL fluids demonstrated a preponderance of neutrophils and lymphocytes with few if any eosinophils or basophils. By using CD3- ζ as a normalizing gene we were able to account for the contribution of these cells to cytokine mRNA production in the BAL samples as its expression was directly correlated with the numbers of T cells in the samples. An in situ hybridization study also identified lymphocytes as the likely source of cytokine mRNA in the lungs of RAO-affected horses (Lavoie et al., 2001).

The lack of IL-5 in the affected horses was consistent with the absence of an eosinophilia in equine RAO (Dixon et al., 1995c; Seahorn et al., 1997). In horses with RAO the predominant cellular response in the airway is a neutrophilia (Dixon et al., 1995c; Franchini et al., 1998; McGorum and Dixon, 1993). Though most studies of human asthma emphasize the presence of eosinophils in bronchial airway wall (Hamelmann et al., 1999), elevated concentrations of IL-8 and increased numbers of neutrophils are found in patients with chronic asthma (Vrugt et al., 1996). Thus, the increased amount of IL-8 and IFN- γ in the airway secretions from patients with asthma, and horses with COPD (Ainsworth et al., 2003; Franchini et al., 1998; Tsoumakidou et al., 2004) may be markers of an ongoing inflammatory process that is more pronounced in those patients with the chronic form of the disease (Nocker et al., 1996). In general, equine RAO is a disease of older horses with the average age at diagnosis being greater than 8 years (Dixon et al., 1995a; Leguillette,

2003). It is likely that younger horses are affected prior to diagnosis but clinical signs are less severe. While it has been proposed that inflammatory airway disease in the young horse may be a precursor to RAO in the older horse (Viel, 1997), there are no experimental data to support this theory. Nevertheless, it is likely that horses in the earliest stage of RAO exhibit a classic Type 2 cytokine response to the allergen. Increased production of the cytokines IL-4 and IL-13 from both T and non-T cells occur following antigen exposure and the triggering of IgE-armed cells. The release of various mediators including chemokines and pro-inflammatory cytokines gives rise to an inflammatory response. With prolonged exposure to the allergen, this response enters a chronic phase characterized by increased production of IL-8 and IFN- γ . Airway remodeling likely occurs during this stage and the horse begins to present with signs of classic “heaves” (Costa et al., 2000; Kaup et al., 1990a,b). In this regard, RAO in horses shares characteristic pathophysiologic features with chronic asthma in humans (Bousquet et al., 2000; Jeffery, 1994; Tsoumakidou et al., 2004). Asthma in humans and mouse models is immunologically mediated and the Type 2 cytokines IL-4 and IL-13 play a central role by favoring IgE antibody production towards inhaled allergens (Del Prete, 1992; Erb and Le Gros, 1996; Hogg, 1997; Wills-Karp, 1999). In human asthma, there is increasing evidence that the production of IFN- γ also contributes to the pathology, particularly in the chronic forms of the disease (Ford et al., 2001; Lukacs et al., 1996; McMillan and Lloyd, 2004). Similarly, RAO-affected horses produce both Types 1 and 2 cytokines depending upon the stage of their disease and the timing of sample collection.

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