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## A novel model for equine recurrent airway obstruction

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### Abstract

Equine recurrent airway obstruction (RAO; a term combining both chronic obstructive pulmonary disease (COPD) and summer pasture associated obstructive pulmonary disease (SPAOPD)) is one of the most common equine respiratory diseases with up to 50% of horses affected worldwide. The etiopathogenesis of RAO is unknown although pulmonary hypersensitivity to inhaled mold antigens may be involved. Recent work in our laboratory demonstrating elevated levels of IL-4 and IL-13 mRNA in the airways and peripheral blood of horses with RAO is consistent with an atopic component to RAO. Little is known regarding the earliest phases of RAO in horses. Here we describe the development of a novel airway model for equine RAO that utilizes ovalbumin-coated polystyrene beads for airway sensitization and challenge. Aerosol challenge of sensitized ponies with OVA-coated microbeads resulted in decreased airway compliance, increased percentage of lymphocytes and neutrophils in the bronchoalveolar lavage fluid, and evidence of a Th2 cytokine response in the bronchoalveolar cells. These results suggest that this approach may be useful in describing the initial stages of RAO development in the horse. © 2002 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Recurrent airway obstruction (RAO) or “heaves” is one of the most commonly diagnosed conditions affecting the equine lung. Two forms of this disease have been identified based on seasonal occurrences: chronic obstructive pulmonary disease (COPD), which occurs primarily in the winter months in stabled horses (Robinson et al., 1995), and summer

pasture-associated obstructive pulmonary disease (SPAOPD), which occurs while horses are on pasture (Seahorn et al., 1996). Similar clinical signs and cellular composition of bronchoalveolar lavage fluid suggest a common disease mechanism. Likewise the anamnestic and reversible nature of equine RAO is similar to some forms of human asthma suggesting a common immunological basis. The current immunological model for human asthma implicates T-helper 2 (Th2) cells as the primary source of the cytokines interleukin (IL)-4, IL-5, and IL-13 that are implicated in the pathophysiologic response to inhaled allergens. While several different pathways may lead to the

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inflammatory changes and nonspecific airway responsiveness underlying asthma, it is clear that Th2 cells and other leukocytes play a central role in its development.

Based on similarities between human asthma and equine RAO, it is likely that Th2 cells and their cytokines would play a significant role in this disease of horses. Thus there is evidence that horses produce IgE antibodies to inhaled allergens associated with COPD (Halliwell et al., 1993; Schmallenbach et al., 1998). Likewise allergen-induced degranulation of basophil and mast cells has been reported (Dirscherl et al., 1993; Hare et al., 1998). There is also evidence of T cell recruitment into the lungs of affected horses (Watson et al., 1997). More recently, we and others have analyzed both bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells from horses with various forms of equine RAO for cytokine-specific mRNA. Recent work in our laboratory demonstrates elevated levels of IL-4 and IL-13 mRNA in the airways and peripheral blood of horses with RAO. This observation is consistent with the idea of a Th2 response being involved in the pathogenesis of RAO.

Little is known regarding the earliest phases of RAO in horses. Here we describe the development of a novel model for equine RAO that utilizes ovalbumin-coated polystyrene beads for airway sensitization and challenge. Aerosol challenge of sensitized ponies with OVA-coated microbeads resulted in decreased airway compliance, an increase in lymphocytes and neutrophils in the bronchoalveolar lavage fluids, and evidence of a Th2 cytokine response in the bronchoalveolar cells. The results demonstrate that this approach may be useful in describing the initial stages of RAO development in the horse.

## 2. Methodology

### 2.1. Analysis of RAO Horses

The population of horses under study included a herd of horses affected with SPAOPD that were sampled when clinical symptoms were present (summer) and absent (winter) (Costa et al., 2000). Control horses pastured with the SPAOD horses were also sampled at the same times. Both BAL and peripheral

blood mononuclear cells (PBMC) were collected for analysis. Total RNA was extracted using routine methods and cytokine-specific mRNA levels were measured using a semi-quantitative PCR assay and equine-specific plasmid curves to determine copy numbers of IL-4, IL-5, IL-13 and IFN- $\gamma$  (Swiderski et al., 1999). The sequence for equine IL-13 was obtained by cross-species PCR amplification using consensus sequence primers based on published IL-13 sequences.

### 2.2. OVA sensitization protocol

Ponies were sensitized by an intramuscular injection of 10 mg of ovalbumin in 225 mg alum. Control ponies were not vaccinated. Two weeks following ovalbumin priming ponies were exposed to aerosolized ovalbumin-coated polystyrene beads. Beads were covalently labeled with ovalbumin according to the manufacturer's protocol (Polysciences, Warrington, PA). Each pony received inhalation (60 min duration) of 14 ml of beads for 5 days per week for 2 weeks. Ponies were rested for 2 weeks and challenged with the same dose of ovalbumin-coated polystyrene beads.

### 2.3. Pulmonary function testing, bronchoalveolar lavage harvest and analysis

Pulmonary function testing was performed 48–72 h post-challenge via forced airway oscillation using a technique similar to that described by Young and Hall (1989). Bronchoalveolar lavage (BAL) samples were also collected at this time. Tranquilized ponies were lavaged following intubation with a sterile stomach tube containing a wide bore catheter. Approximately 60 ml of sterile PBS was passed to the lungs and recovered 4–5 times for a total lavage wash of 300 ml fluid. Flocculent material was allowed to settle out and was discarded prior to centrifugation of fluid to recover BAL cells. The BAL fluid was saved at  $-20^{\circ}\text{C}$  for future cytokine profile analysis and BAL cells were recovered for mRNA analysis.

### 2.4. Quantitative RT-PCR

Cellular RNA was isolated using RNeasy, according to manufacturers suggested protocol (Tel-Test,

Friendswood, TX) and cDNA was synthesized. Cytokine message was quantitated using the real time PCR approach developed by PE Biosystems (Roche, Branchburgh, NJ). Experimental sample cytokine copy number was determined by comparison to the signals generated from a standard curve constructed with plasmids containing the appropriate cytokine cDNA sequence. Cytokine message copy number was normalized to  $\beta$ -actin or CD3 $\zeta$  signal to control for cell number or T cell number, respectively.

### 3. Results

Control animals exhibited a bias towards Th1 cytokine (interferon- $\gamma$ ) production in their lungs throughout the year. Horses affected with SPAOPD exhibited elevated levels of mRNA for IL-4 and IL-13 during the summer months when clinical symptoms were present. While half of the SPAOPD-affected animals returned to a Th1 bias in their mRNA profile in the winter, the other horses exhibited elevated signs of IL-4 and IL-13 mRNA suggesting a more chronic condition in these animals. We failed to detect elevated levels of mRNA for IL-5 in the lungs of affected horses.

Aerosol challenge of ovalbumin-sensitized ponies with ovalbumin-coated microbeads resulted in decreased airway compliance as compared to pre-challenge measurements. Lymphocyte and neutrophil percentages were increased in the cells isolated from bronchoalveolar lavage in comparison to control animals. This increase in neutrophil number is similar to the changes in lung infiltration profile seen in both SPAOPD and COPD afflicted ponies. Higher levels of IL-4, IL-5 and IL-13 mRNA were seen in OVA-primed animals in comparison to control ponies. IFN- $\gamma$  levels in control ponies were comparable for one animal and dramatically increased in the second, in comparison to OVA-primed ponies. While sample numbers were small in this preliminary study, these results suggest that the BAL cytokine production is being skewed to a Th2 cytokine response in comparison to control ponies.

### 4. Discussion

Our results with the SPAOPD horses and the COPD cases were similar to other reports that BAL cells from

COPD horses exposed to dusty hay have increased expression of IL-4 and decreased expression of IFN- $\gamma$  mRNA (Robinson, 2001). This increase in IL-4 mRNA expression in heaves is consistent with the elevated levels of antigen-specific IgE in serum and BAL of affected horses (Halliwell et al., 1993; Schmallenbach et al., 1998). Likewise, our novel description of mRNA for IL-13 in these samples is also consistent with an IgE-mediated response. While IL-4 and IL-13 were thought to play overlapping roles in regulating B cell switching to IgE, recent results using transgenic mice suggest IL-13 may be more important in the induction of anaphylaxis through its effect on IgE production (Fallon et al., 2001). Both IL-4 and IL-13 have other pathophysiologic effects in asthma that are IgE-independent (Doucet et al., 1998; Wills-Karp et al., 1998; Luttmann et al., 1999; Li et al., 1999). In particular, transgenic IL-13 expression was associated with a mononuclear and eosinophilic inflammatory response, mucus cell metaplasia, airway fibrosis, eotaxin production, airway obstruction, and nonspecific airway hyper-responsiveness (Zhu et al., 1999). Both IL-4 and IL-13 were detected in the equine RAO samples, though levels of IL-13 mRNA were consistently lower than those of IL-4. While we do not know how this corresponds to actual protein levels, given its potent biological activity low level transcription of equine IL-13 could still account for many of the physiologic changes associated with equine RAO.

Our failure to detect elevated levels of mRNA for IL-5 in the lungs of affected horses is also consistent with the absence of eosinophils in the bronchoalveolar samples of RAO horses (Wardlaw, 1999). Whereas human asthma is characterized by a 50- to 100-fold increase in the number of eosinophils relative to neutrophils in the bronchial mucosa, this is not the case in equine RAO. IL-8, which functions as a chemokine for both neutrophils and eosinophils (Erger and Casale, 1995), is present in equine BALF (Franchini et al., 1998). The central role IL-5 plays in the induction of eosinophilia and its apparent absence in equine RAO appears to result in a predominantly neutrophilic infiltration in this disease. What role eosinophils play in the asthmatic response is also uncertain given the similarities of symptoms between human asthma and equine RAO. Indeed this calls into question the rationale of targeting IL-5 as a potential therapeutic approach to human asthma (Garlisi, 1999).

Why IL-5 is not produced in equine RAO while other Th2 cytokines (IL-4 and IL-13) are produced is not known. The regulation of IL-5 production in humans and mice has only recently been described (Blumenthal et al., 1999; Zhang et al., 1998 and Nakamura et al., 1999). Like most cytokines, IL-5 is regulated at the transcriptional level, though its transcription is dependent upon de novo protein synthesis (Naora and Young, 1995). Results in humans and mice are consistent with our IL-5 mRNA results and indicate that IL-5 and the other Th2 cytokine genes are independently regulated in equine RAO. Further characterization of the molecular mechanisms regulating the expression of these genes seems warranted.

The pre-disposing factors for the induction of equine RAO remain uncertain. Since most clinical cases present as a late/chronic stage of the disease it is not known whether earlier processes in disease development might have a different phenotype. While it has been proposed that inflammatory airway disease in the young horse might predispose these animals for RAO (Viel, 1997), such a relationship has not been directly demonstrated (Robinson, 2001). Characterization of the cytokine profile and antigen-reactivity of these younger horses might prove useful in understanding this relationship. Alternatively, employment of an RAO model will allow characterization of immune-mediated events that precede clinical presentation of RAO and will further our understanding of this disease. In addition, development of an RAO model will allow examination of the immune events during a primary exposure in naive animals. Understanding the immune events preceding symptomatic disease may lead to improved therapeutic options. While the results reported here are preliminary, they strongly suggest that challenge of OVA-primed ponies with aerosolized microbeads will yield a viable model for RAO. The evidence of increased respiratory impedance, combined with neutrophilia and increased Th2 cytokine transcription is consistent with the Th2 nature of the disease. Interestingly, one control animal which did not receive an intramuscular OVA-priming injection, but did receive aerosolized OVA-coated beads, showed evidence of mild respiratory impedance upon OVA challenge suggesting that the pony responded to the bead priming protocol in the absence of intramuscular priming. This is currently under investigation, and may lead to the development of

an aerosol-only priming model more similar to the presumed natural priming route. Further development of this model should enable us to characterize the early induction stages and progression of immune processes leading to the development of obstructive pulmonary disease.

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