

Interleukin-4 and interferon-gamma gene expression in summer pasture-associated obstructive pulmonary disease affected horses

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Keywords: horse; SPAOPD; RAO; IL-4; IFN- γ ; T helper cell

Summary

We hypothesised that horses affected with summer pasture-associated obstructive pulmonary disease (SPAOPD) react to an allergen or allergens in their summer environment that is either absent or present at lower levels in their winter environment; and that such allergens stimulate SPAOPD-affected horses to produce a different T helper lymphocyte cytokine profile from that of control horses. The primary objective of this study was to determine the cytokine mRNA profile of T helper lymphocytes obtained from summer pasture-associated obstructive pulmonary disease (SPAOPD) affected horses when 1) the horses were showing signs of disease (summer) and 2) they were in clinical remission (winter). A further objective was to determine the differences between cytokine mRNA T helper lymphocyte profiles of control and affected horses in the summer and winter seasons. Interleukin 4 (IL-4) and interferon-gamma (IFN- γ) mRNA expression levels were increased in bronchoalveolar lavage fluid (BALF) and peripheral blood mononuclear cell (PBMC) samples of affected horses during disease expression. No significant amounts of IL-5 mRNA were detected in any of the samples. These results suggest that there is an allergic component to SPAOPD of horses and that appropriate manipulation of the immune system could offer hope for treatment and prevention of the disease in the future. Further research studies will be needed to determine the most appropriate treatments to use to alter the antigen-stimulated cytokine profile being expressed by SPAOPD-affected horses or to alter the effects that these cytokines produce.

Introduction

Summer pasture-associated obstructive pulmonary disease (SPAOPD) is a disease of horses that is characterised by recurrent airway obstruction (RAO). The disease was so named because of the proclivity of horses grazing on pasture in the hot and humid south-eastern United States to develop clinical signs of the disease in the summer (Beadle 1983). More recently, a similar disease has been reported in Scotland (Dixon and McGorum 1990) and

England (Mair 1996). Pathophysiological characteristics of the disease include airway inflammation, increased airway mucus production and bronchoconstriction.

The mechanism whereby the above noted changes are induced in the airways of affected horses is currently not known. However, it is suspected that an allergen from the environment could be causing an immunologically-induced response in the airways of affected horses. In this regard, it is felt that the pathogenesis of SPAOPD could be very similar to that of asthma in man.

Activated CD4⁺ cells (T helper lymphocytes) are found in increased numbers in BALF and mucosal samples collected from the airways of human asthmatics (Bradley *et al.* 1991; Gerblich *et al.* 1991; Bentley *et al.* 1992; Walker *et al.* 1992). Additionally, cytokine profiles indicate that there is a switching from the TH₁ subset of CD4⁺ cells, which produces interleukin-2 (IL-2) and IFN- γ , to the TH₂ subset, producing IL-4 and IL-5, in the airways of human asthmatics (Robinson *et al.* 1992, 1993; Ying *et al.* 1995). Since T helper lymphocytes are felt to play a critical role in the development of human asthma, this project was undertaken to see if T helper lymphocytes play a role in the pathogenesis of SPAOPD in horses.

Materials and methods

This study was approved by the Animal Care and Use Committee of Louisiana State University and conducted under its guidelines.

Affected horses

Six mature horses of various breeds (4 mares and 2 geldings), diagnosed previously as being affected with SPAOPD, served as the affected group. These horses were maintained at the School of Veterinary Medicine on a year-round basis. They were vaccinated annually and administered anthelmintics 3 times/year. They were maintained on pasture during the project and grazed native grasses and ryegrass during the summer and winter seasons, respectively. A pelleted complete horse feed (Horse Chow 100)¹ was fed as necessary to maintain good body condition.

Control horses

Three mature Thoroughbred geldings served as the control group.

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TABLE 1: Bronchoalveolar fluid (BALF) and peripheral blood mononuclear cell (PBMC) cytokines. Minimums, maximums and medians are expressed as mRNA copy units for the appropriate cytokine

Disease group		Winter	Summer	P value	
<i>BALF IL-4</i>					
Control	Minimum	1.7E+3	2.8E+3	0.074	
	Maximum	2.8E+3	9.8E+3		
	Median	2.1E+3	3.8E+3		
SPAOPD	Minimum	1.5E+3	1.0E+4		0.910
	Maximum	8.3E+5	8.5E+4		
	Median	1.2E+4	5.9E+4		
P value		0.655	0.04*		
<i>PBMC IL-4</i>					
Control	Minimum	2.5E+3	7.6E+3	1.000	
	Maximum	3.0E+5	2.2E+4		
	Median	2.8E+3	7.8E+3		
SPAOPD	Minimum	1.3E+3	2.1E+4		0.065
	Maximum	4.3E+4	2.1E+5		
	Median	1.0E+4	5.6E+4		
P value		0.655	0.040*		
<i>BALF IFN-γ</i>					
Control	Minimum	2.4E+3	5.0E+5	0.074	
	Maximum	6.3E+3	1.7E+6		
	Median	4.1E+3	9.2E+5		
SPAOPD	Minimum	3.9E+3	1.4E+6		0.013*
	Maximum	3.1E+5	1.6E+7		
	Median	8.5E+3	3.3E+6		
P value		0.655	0.040*		
<i>PBMC IFN-γ</i>					
Control	Minimum	3.4E+3	9.0E+5	0.074	
	Maximum	9.4E+4	3.2E+6		
	Median	6.3E+3	1.4E+6		
SPAOPD	Minimum	5.4E+3	1.7E+6		0.013*
	Maximum	2.0E+4	1.1E+7		
	Median	1.4E+4	5.2E+6		
P value		0.655	0.040*		

*Statistical significance at P = 0.05 level.

These horses were free of any clinically evident respiratory disease and had no indication of chronic or recurrent respiratory disease in their historical record. They were maintained in the same pasture environment as the affected group of horses and were fed similarly.

Schedule of studies and duration

Data were collected during the summer when horses in the affected group were showing distinct signs of airway obstruction while on pasture (i.e. clinical score 4.5) and again in February when they were in disease remission. This allowed examination of data from both groups of horses during the zenith and nadir of the clinical syndrome in the affected group. We attempted to eliminate any diurnal variations in the data (Stadler and Deegen 1986) by studying the horses between 10:00 and 12:00 h in summer and winter. Horses were removed from pasture for sample collection but were returned immediately thereafter.

Clinical evaluation

Medial and lateral nostril flare (MNF, LNF) during inspiration and abdominal lift (AL) during exhalation were evaluated to determine a clinical score (CS) for severity of the obstructive disease present in these horses.

For MNF and LNF, 0 = no or very slight movement of nostril aspect noted on inspiration; 1 = nostril aspect flared only slightly during inspiration and returned to a normal position as inspiration ended; 2 = nostril aspect flared during inspiration and returned to a near normal position as inspiration ended; 3 = nostril aspect flared to a greater extent during inspiration and did not approach a normal position as inspiration ended or during exhalation; and 4 = nostril aspect was flared maximally and remained so throughout the respiratory cycle.

For AL, 0 = no or very slight movement in the ventral flank; 1 = slight abdominal flattening present with a 'heave line' just beginning to form in the cranial aspect of the ventral flank; 2 = abdominal flattening was obvious and a 'heave line' was present and extending to, but not beyond, a point halfway between the *tuber coxae* and the point of the elbow; 3 = abdominal flattening was obvious and a 'heave line' extended beyond a point halfway between the *tuber coxae* and the point of the elbow but did not extend to the elbow; 4 = abdominal flattening was obvious and a 'heave line' extended cranially to the elbow. The clinical score was determined from the following algorithm:

$$CS = (MNF + LNF)/2 + AL$$

In addition, rectal temperature, respiratory rate and heart rate were determined for each animal immediately prior to data collection.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed using a lodged-catheter technique as previously described (Rossier *et al.* 1991). The lavage procedure used sterile phosphate-buffered saline and the first 150 ml recovered by aspiration were placed in sterile tubes and used for analysis. All horses were bronchodilated and sedated before undergoing this procedure. Bronchodilation was achieved using 0.0022 mg/kg bwt glycopyrrolate (Robinul-V)² i.v. Intravenous xylazine (Rompun)³ (150 mg) and butorphanol (Torbugesic)² (5 mg) were used as sedative agents. Cytology was performed on each BAL sample collected.

Haematology

Ten ml peripheral blood samples were collected aseptically from the jugular vein into EDTA-containing vacuum tubes. Haematology was performed on each sample of venous blood collected.

Lymphocyte preparations

Forty ml heparinised 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 (PBS), underlayered with ficoll-hypaque (Ficoll-Paque)⁴ and centrifuged at 800 g for 30 min (Swiderski *et al.* 1999a). Peripheral blood mononuclear cells

TABLE 2: Bronchoalveolar fluid (BALF) nucleated cells. Minimums, maximums and medians are percentages of total cells

Disease group		Winter	Summer	P value
<i>Neutrophils</i>				
Control	Minimum	0	14	0.074
	Maximum	5	18	
	Median	0	16	
SPAOPD	Minimum	0	11	0.006**
	Maximum	11	94	
	Median	2.5	60	
P value		0.655	0.074	
<i>Mast cells</i>				
Control	Minimum	8	5	0.074
	Maximum	40	8	
	Median	15	6.5	
SPAOPD	Minimum	1	0	0.006**
	Maximum	14	2	
	Median	5	0.5	
P value		0.025*	0.025*	
<i>Macrophages</i>				
Control	Minimum	40	60	0.074
	Maximum	50	70	
	Median	46	65	
SPAOPD	Minimum	1	0	0.064
	Maximum	67	40	
	Median	52.5	10	
P value		0.204	0.025*	
<i>Lymphocytes</i>				
Control	Minimum	10	11	0.383
	Maximum	52	13	
	Median	30	12	
SPAOPD	Minimum	0	0	0.285
	Maximum	34	26	
	Median	8	3.5	
P value		0.371	0.025*	
<i>Epithelial cells</i>				
Control	Minimum	0	0	0.795
	Maximum	4	1	
	Median	0	0.5	
SPAOPD	Minimum	0	0	0.009**
	Maximum	93	89	
	Median	21.5	0	
P value		0.074	0.371	

*Statistical significance at P= 0.05 level; **Statistical significance at P = 0.01 level.

(PBMC) were subsequently extracted from the interface layer, washed in PBS and counted. A mixed population of nucleated cells including lymphocytes was obtained from bronchial lavage 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 blue⁵ exclusion dye to assess viability.

Quantitative polymerase chain reaction analysis of cytokine production

For quantitative polymerase chain reaction (QPCR) analysis of cytokine production, 3×10^6 BALF cells or PBMC were placed into an acid guanidium thiocyanate-phenol disassociation buffer (RNA Stat-60)⁶ and frozen. Frozen samples were quickly thawed and the RNA extracted using a phenol:chloroform procedure. The RNA samples were processed for the analysis of cytokine-specific mRNA using the RT-PCR procedure for equine cytokines (Swiderski *et al.* 1999a). Total cellular RNA was isolated using a phenol:chloroform procedure (Chomczynski and Sacchi 1987) and reverse transcribed into cDNA using MMLV reverse transcriptase and oligo dT primers. A quantitative PCR (QPCR) method using a semi-automated QPCR System 5000 was used to analyse PCR products (Wilkinson *et al.* 1995).

Statistics

Because the data were not normally distributed, nonparametric tests were used to analyse the results for statistical significance. Wilcoxon's signed rank test was used to analyse for the presence of a seasonal effect in data that were collected from horses in the same group in different seasons, while the median test was used to test for a disease effect in data that were collected from affected and control horses in the same season (Steel and Torrie 1980). Statistical significance was set at P = 0.05.

Results

Cytokine analyses

Results of the IL-4 and IFN- mRNA analyses are contained in Table 1. The highest median levels of IL-4 mRNA were detected in summer samples obtained from affected horses. These increased IL-4 mRNA levels were seen in samples obtained from BALF as well as in samples obtained from peripheral blood mononuclear cells (PBMC). BALF IL-4 mRNA levels were significantly higher (P = 0.040) in affected compared to control horses for these summer samples, as were those for PBMC IL-4 mRNA (P = 0.040).

Similar to the IL-4 mRNA results, the highest median levels for IFN- mRNA were detected in summer samples obtained from affected horses. Again, this included IFN- mRNA detected in both BALF and PBMC samples taken from affected horses. IFN- mRNA levels detected in the BALF summer samples obtained from affected horses were significantly (P = 0.040) higher than the IFN- mRNA levels detected in the BALF summer samples obtained from control horses and significantly different (P = 0.013) from the BALF winter samples obtained from affected horses. IFN- mRNA levels detected in the PBMC summer samples obtained from affected horses were significantly (P = 0.04) higher than the IFN- mRNA levels detected in the PBMC summer samples obtained from control horses and they were significantly different from the PBMC winter samples obtained from affected horses (P = 0.013).

Summer and winter BALF and PBMC samples from both control and affected horses were analysed to determine if IL-5 mRNA transcripts were being produced by lymphocytes in these samples. No significant amounts of IL-5 mRNA were detected in any of these samples.

BALF cytology

Percentages of nucleated cells present in BALF samples are presented in Table 2. There was a significantly higher percentage of neutrophils present in summer as compared to winter samples collected from affected horses ($P = 0.006$). A significantly lower percentage of mast cells was present in summer as compared to winter samples collected from affected horses ($P = 0.006$). In addition, there were significantly larger percentages of mast cells in BALF of control horses compared to affected horses in both summer ($P = 0.025$) and winter ($P = 0.025$) samples.

Macrophage and lymphocyte median percentages were lowest in BALF samples collected from affected horses during the summer season. These percentages were significantly decreased from those of control horses in the summer season for both macrophages ($P = 0.025$) and lymphocytes ($P = 0.025$). Finally, there was a significantly higher percentage of columnar epithelial cells present in winter as compared to summer BALF samples collected from affected horses ($P = 0.009$).

Haematology

Statistical analysis of haematology data showed that summer neutrophil absolute counts of affected horses were significantly higher ($P = 0.026$) than those of affected horses in the winter.

Clinical variables

The highest values for CS were recorded for affected horses during the summer sampling season. These values were significantly elevated over those for control horses during the summer season ($P = 0.005$) and those for affected horses during the winter season ($P = 0.004$).

Summer respiratory rates of affected horses were significantly higher than those of affected horses during the winter ($P = 0.013$), as were pulse rates of affected horses ($P = 0.013$). Similarly, the summer body temperatures of affected horses were significantly higher than those of affected horses during the winter ($P = 0.005$). Finally, a statistically significant group effect was seen in the summer respiratory rates, with the rates of control horses being higher than those of affected horses ($P = 0.025$).

Discussion

The lack of an IL-5 mRNA response in the affected horses is consistent with the absence of an eosinophilia in equine SPAOPD (Dixon and McGorum 1990; Seahorn *et al.* 1997; Costa *et al.* 2000). By contrast, IL-5 has been strongly implicated in the aetiology of asthma because of its pivotal role in eosinophil development and activation (Hogan *et al.* 1998). Human asthmatic patients have increased numbers of eosinophils in their airways whereas, in horses with SPAOPD, the predominant cellular response is the neutrophil (Dixon and McGorum 1990; Mair 1996; Seahorn *et al.* 1997; Costa *et al.* 2000). Although most studies of human asthma emphasise the presence of eosinophils in the bronchial airway wall, elevated levels of IL-8 and increased numbers of neutrophils are found in patients with unstable asthma (Vrugt *et al.* 1996). Therefore, the increased levels of IL-8 in the airway secretions from patients with asthma, and horses with COPD (Franchini *et al.* 1998), may be markers of an ongoing inflammatory process which is more pronounced in those patients

with more severe disease (Nocker *et al.* 1996).

The dramatic rise in BAL (4.9-fold) and PBMC (5.6-fold) IL-4 mRNA transcripts in summer as opposed to winter samples obtained from affected horses is consistent with a hypothesis that affected horses were reacting to an allergen or allergens in their summer environment that was not present or was present at lower levels in their winter environment, and that such allergens were stimulating TH₂ lymphocytes to produce IL-4. Because the increase in IL-4 mRNA transcripts was seen in both PBMC and BALF samples, it appears that the response was systemic as well as local. The fact that the summer BAL IL-4 response seen in control horses was significantly different from that seen in affected horses leads to the conclusion that these allergens were not stimulating the TH₂ lymphocyte population of control horses to such a degree as those of affected horses.

The cytokine IL-4 plays several key roles in the development of asthmatic disease and allergies. Studies of murine models of asthma have demonstrated that IL-4 plays a central role in establishing the cascade of responses required to generate airway hyperactivity to inhaled antigen (Corry *et al.* 1996; Wills-Karp and Ewart 1997). Interleukin-4 is also involved in the preferential activation of TH₂ cells and the subsequent suppression of TH₁ cells (Del Prete 1992; Anderson and Coyle 1994) and controls the production of IgE antibodies (Zhou *et al.* 1997). The IL-4 gene cluster has also been genetically linked to asthma (Casillas and Nel 1997). Together, these results indicate a central role for this cytokine in human asthma and our results imply that it may also be an important factor in equine SPAOPD.

Evidence exists that TH₂ cytokine responses in ponies are associated with a protective immunity to *Strongylus vulgaris* (Swiderski *et al.* 1999b). This protection was correlated with the production of mRNA for TH₂ cytokines IL-4 and IL-5 in PBMC and colonic lymph nodes of the protected ponies. By contrast, the immunopathological response in equine recurrent uveitis is associated with a TH₁ cytokine response (Gilger *et al.* 1999). Together, these and other results indicate that horses can mount classical TH₁ and TH₂ immune responses (Horohov 2000).

In spite of the presence of increased levels of IL-4 mRNA in the affected horses, it seems unlikely that SPAOPD represents a classic TH₂ response, since we failed to detect mRNA for IL-5 in these same samples. In human patients, with allergic asthma, the cytokine pattern is compatible with a pure TH₂ response (elevated IL-4 and IL-5) (Virchow *et al.* 1996). There is evidence for selective production of either IL-4 or IL-5 in response to different immune stimulation and pathological situations (Sewell and Mu 1996), such as intrinsic asthma, which is characterised by elevated IL-5 and IL-2 but not IL-4 (Virchow *et al.* 1996).

Although the genes encoding IL-4 and IL-5 are located on the same chromosome in man and mice, their orientation is reversed (Kotsimbos and Hamid 1997) and their expression appears to be regulated differentially (Naora and Young 1995). Little is known regarding the regulation of IL-4 and IL-5 gene expression in horses, although our results would suggest a similar disassociation of regulatory elements.

Coincident with the IL-4 mRNA response in affected horses in the summer season, there was also an increase in the IFN- mRNA transcripts in these same samples. This finding indicates that there was not a TH₁:TH₂ isotype switching occurring in these horses but rather that both TH₁ and TH₂ lymphocyte populations were being simultaneously stimulated. The IFN- mRNA summer vs. winter response in control horses was muted as compared to affected

horses but did appear to be present to a smaller degree.

While the precise cellular source of the cytokine mRNA was not determined, horses affected with COPD exhibit elevated levels of CD4⁺ lymphocytes in the peripheral blood and CD3⁺ cells in pulmonary biopsy frozen tissue sections (Watson *et al.* 1997). As noted above, cytopsin analysis of BALF fluids from this study demonstrated a preponderance of neutrophils and lymphocytes with no eosinophils or basophils. While multiple cell types, including basophils and eosinophils, can produce IL-4 (Kuna *et al.* 1996), it appears probable that the cytokine mRNA measured in this study was produced by the lymphocytes, especially since similar levels were also seen in the PBMC samples which are devoid of granulocyte contamination. Nevertheless, subsequent characterisation of the source of the cytokine mRNA in BALF is warranted.

The statistically significant increase in the percentage of neutrophils in the summer as compared to winter BALF samples is confirmatory evidence that affected horses were undergoing an episode of RAO at the time of sampling. This finding was congruous with the statistically significant increase in the summer vs. winter CS for these same horses. Elevated neutrophil counts seen in the summer haematology findings of affected horses are thought to be a mild yet significant reflection of the neutrophilia occurring in the airways of these horses. A rise in the percentage of neutrophils in the summer as compared to winter BALF samples of control horses, even it did not reach the levels seen in summer BALF samples of affected horses, was noted. This rise is similar to that seen when control horses were exposed to an environment that elicited clinical signs in COPD horses (Tremblay *et al.* 1993). SPAOPD and COPD are precipitated by different environmental circumstances but both are characterised by RAO in horses. Additional studies are indicated to further characterise and elucidate this common response in control horses to distinctly different environments.

The precise reason for a statistically significant disease effect seen in the summer BALF macrophage and lymphocyte percentages was not investigated. Because the BALF macrophage and lymphocyte percentages rose during periods of disease remission, i.e. in winter BALF samples, the summer decreases are thought to be dilutional and caused by a dramatic influx of neutrophils into the airways of SPAOPD horses showing clinical signs of disease expression. The lowest percentage of BALF mast cells was seen in the summer samples of affected horses. The high number of summer BALF neutrophils in affected horses would tend to decrease the percentage of BALF mast cells as was noted above for macrophages and lymphocytes. However, it is enticing to speculate that the mast cells were also being degranulated and, therefore, rendered unrecognisable as part of an IL-4-stimulated and IgE-mediated allergic response occurring in the airways of affected horses during the summer season. Further studies are needed to determine the contribution of these 2 possibilities.

An explanation for the statistically significant decrease in the percentage of summer as compared to winter BALF epithelial cells of affected horses is equivocal. In addition to the dilutional effect of an influx of neutrophils into the airways, another possible explanation is that the summer BALF samples were collected from a decreased surface area of accessible epithelial cells as compared to the winter samples due to the presence of airway obstruction when the summer samples were taken.

The reasons for the elevations in respiratory rate, pulse rate and body temperature for affected horses during disease

expression were not investigated. However, hypoxaemia and/or increased work of breathing could be possible explanations for the increases in each of these variables.

The finding from this study that affected horses express higher levels of IL-4 mRNA and IFN- mRNA in their BALF cells and PBMC during disease exacerbations has important clinical implications. In view of this detected increase in cytokine expression, it may be beneficial to manipulate the immune systems of affected horses to prevent them from expressing these higher levels of IL-4 mRNA and IFN- mRNA or to prevent the resultant cytokines from interacting with their receptors. If such therapy were successful, a major step forward would have been accomplished in the prevention and treatment of SPAOPD in horses.

Acknowledgements

The authors thank Keiko Antoku, Kim Snedden, Chad Vanderheyden, Britta Leise, Susan Porciau, Paul Hollier and Frank Garza for their technical assistance. Supported by an Equine Veterinary research grant from the School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803, USA and a research grant from the Grayson-Jockey Club Research Foundation, Inc., Lexington, Kentucky 40503, USA.

Manufacturers' addresses

- ¹Purina Mills, Inc., St. Louis, Missouri, USA.
- ²Fort Dodge Animal Health, Fort Dodge, Iowa, USA.
- ³Bayer Corp., Agriculture Division, Animal Health, Shawnee Mission, Kansas, USA.
- ⁴Pharmacia LKB Biotechnology, Piscataway, New Jersey, USA.
- ⁵Sigma Chemical Company, St. Louis, Missouri, USA.
- ⁶Tel-Test, Friendswood, Texas, USA.

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Received for publication: 6.7.00

Accepted: 19.6.01