



## Cytokine profiles of peripheral blood and airway CD4 and CD8 T lymphocytes in horses with recurrent airway obstruction

C. Kleiber<sup>a,\*</sup>, B.C. McGorum<sup>b</sup>, D.W. Horohov<sup>c</sup>, R.S. Pirie<sup>b</sup>,  
A. Zurbriggen<sup>d</sup>, R. Straub<sup>a</sup>

<sup>a</sup>*Department of Clinical Veterinary Medicine, University of Berne, Länggasstrasse 124,  
3012 Berne, Switzerland*

<sup>b</sup>*Wellcome Trust Centre for Research in Comparative Respiratory Medicine,  
Easter Bush Veterinary Centre, University of Edinburgh, Roslin, Midlothian EH25 9RG, UK*

<sup>c</sup>*Department of Veterinary Science, University of Kentucky, Maxwell H. Gluck Equine Research Center,  
Lexington, KY 40546-0099, USA*

<sup>d</sup>*Department of Clinical Veterinary Medicine, Division of Clinical Research, University of Berne,  
Bremgartenstrasse 109A, 3012 Bern, Switzerland*

Received 23 December 2003; received in revised form 16 June 2004; accepted 19 August 2004

### Abstract

Equine recurrent airway obstruction (RAO) is thought to result from an aberrant immune response to inhaled antigens, modulated by T lymphocytes via the secretion of pro-inflammatory cytokines. However data relating to the phenotypes of the T lymphocytes present in peripheral blood and bronchoalveolar lavage fluid of RAO horses and their cytokine profiles are contradictory. The aim of this study was to further investigate the cytokine (IL-4, IL-5, IL-13 and INF- $\gamma$ ) mRNA expression profile in peripheral blood lymphocytes and bronchoalveolar lavage lymphocytes from RAO and control horses, before and at 48 h after horses were exposed to hay/straw. In contrast to previous studies, cytokine expression was quantified in populations of CD4 and CD8 T lymphocytes which were purified using magnetic bead antibody cell separation. Hay/straw exposure induced clinical airway obstruction, airway neutrophilia and airway lymphocytosis in RAO horses, and, induced a mild, but significant, airway neutrophilia in controls. However, hay/straw exposure had no significant effect on peripheral blood lymphocyte or bronchoalveolar lavage lymphocyte cytokine expression in either group. In conclusion, RAO was not associated with alterations in lymphocyte cytokine expression that are consistent with Th1 or Th2 responses, but rather with a general down-regulation in expression of the measured cytokines in peripheral blood lymphocytes and bronchoalveolar lavage lymphocytes.

© 2004 Elsevier B.V. All rights reserved.

**Keywords:** Cytokine; T lymphocytes; Quantitative polymerase chain reaction; Recurrent airway obstruction (RAO); Heaves; Horse

**Abbreviations:** BALF, bronchoalveolar lavage fluid; BALL, bronchoalveolar lavage lymphocytes; RAO, recurrent airway obstruction; SPAOPD, summer pasture-associated obstructive pulmonary disease

\* Corresponding author. Tel.: +41 31 631 22 43; fax: +41 31 631 26 20.

E-mail address: [claudia.kleiber@knp.unibe.ch](mailto:claudia.kleiber@knp.unibe.ch) (C. Kleiber).

## 1. Introduction

Equine recurrent airway obstruction (RAO) is thought to result from an aberrant immune response to inhaled antigens, modulated by T lymphocytes via the secretion of pro-inflammatory cytokines (Giguere et al., 2002; Lavoie et al., 2001). However data relating to the phenotypes of the T lymphocytes present in PB and bronchoalveolar lavage fluid (BALF) of RAO horses (Kleiber et al., 1999; McGorum et al., 1993; Watson et al., 1997) and their cytokine profiles are contradictory. Lavoie et al. (2001) and Cordeau et al. (2002) found that bronchoalveolar lavage lymphocytes (BALL) from RAO horses had a Th2 cytokine profile, with increased IL-4 and IL-5 mRNA expression and reduced IFN- $\gamma$  expression. Giguere et al. (2002) found that BALL from RAO horses had a mixed cytokine response, with consistently increased IL-8, IL-1 $\beta$  and TNF- $\alpha$  expression, but also with increased IL-4 and IFN- $\gamma$  expression at various times during the study (Giguere et al., 2002). Ainsworth et al. (personal communication) found that 24 h hay/straw exposure had no significant effect on BALL cytokine expression in RAO horses, except for increased IL-8 expression. The aim of this study was to further investigate the cytokine (IL-4, IL-5, IL-13 and INF- $\gamma$ ) profile of PBL and BALL from RAO and control horses, before and at 48 h after horses were exposed to hay/straw. In contrast to previous studies, cytokine expression was quantified in populations of CD4 and CD8 T lymphocytes that had been purified using magnetic bead antibody cell separation. This was done to overcome the problem that differences in cytokine mRNA expression in PBL and BALL may result from differences in the proportions of lymphocytes sub-populations in samples (Giguere et al., 2002).

## 2. Materials and methods

### 2.1. Horses

Five healthy female control horses (median age 6 years, range 4–9; weight 320 kg, 316–356 kg) with no detectable respiratory disease, and 7 horses (3 geldings, 4 mares; age 17, 8–28 years; weight 434 kg, 323–594 kg) with a history and clinical

diagnosis of RAO were studied. The disease status of all subjects was confirmed by hay/straw challenges, as previously described (Brazil, 2000; McGorum et al., 1993; Pirie et al., 2003, 2001). Prior to the study all horses were kept in a low dust environment.

### 2.2. Hay/straw challenge

For the hay/straw challenge, horses were housed for 48 h in a poorly ventilated stable, fed a mixture of good quality hay and hay with visible mould growth, and bedded on deep litter straw. This environment has previously been shown to induce airway inflammation and dysfunction in RAO horses (McGorum et al., 1993). The response to challenges was determined using clinical scoring, BALF cytology, respiratory mechanics analysis and airway reactivity assessment, as previously described (McGorum and Dixon, 1992).

### 2.3. Sample collection

BALF was collected endoscopically as previously described (Pirie et al., 2001). 100 ml of PB was collected by jugular venipuncture into 50 ml tubes containing sodium citrate. Baseline PB and BALF samples were collected after the horses had been maintained in a controlled environment. Baseline BALF was collected at least two weeks prior to allergen exposure to exclude potential inflammatory alterations induced by the lavage procedure per se.

### 2.4. Lymphocyte preparation

To isolate BALL, BALF was filtered through 100  $\mu$ m nylon filters (Falcon, Becton Dickinson, USA), centrifuged at 300  $\times$  g for 5 min, the supernatant discarded and the cell pellet resuspended in PBS. PBL were isolated by ficoll-hypaque separation. Briefly, blood was centrifuged at 1000  $\times$  g to obtain a buffy coat, which was transferred to a new tube, resuspended in saline, underlayered with Ficoll-Paque and centrifuged at 1500  $\times$  g for 30 min. PBL were harvested from the interface layer, washed in PBS and counted.

To detect the origin of the cytokines produced, lymphocyte populations expressing the equine homologue of the CD4 antigen (EqCD4) or the CD8 antigen (EqCD8) were purified using magnetic bead selection.

Briefly, PBMC and BALF cells were resuspended at  $\leq 5 \times 10^6$  per milliliter PBS. To block non-specific binding of the mAbs to Fc receptors human IgG was added at 10  $\mu\text{g/ml}$ . PBL and BALL were then labelled for 45 min with mAbs recognising EqCD4 (HB61) and EqCD8 (HT14), washed with PBS/5 mM EDTA/0.4% FCS and incubated for 30 min with MACS goat anti-mouse immunoglobulin (MiniMACS, Miltenyi Biotec, USA). To determine the purity of the T cell subsets, aliquots of separated cells were incubated an additional 5 min with FITC conjugated goat anti-mouse IgG, washed twice with PBS/5 mM EDTA/0.4% FCS and analysed by flow cytometry. The remainder of the labelled cells were applied to a separation column inside a high gradient magnetic field. The column was washed three times to remove unbound negatively selected cells (depleted cell population) and then removed from the magnetic separation unit to flush out the antibody-labelled positively selected cells (enriched cell population). CD4 and CD8 cell populations were then suspended in lysis buffer, consisting of Buffer RLT (Qiagen) supplemented with 2-mercaptoethanol, at  $3 \times 10^6$  cells/300  $\mu\text{l}$  buffer and frozen at  $-70^\circ\text{C}$ .

### 2.5. RNA isolation, cDNA synthesis, polymerase chain reaction, hybridisation and quantification of the polymerase chain reaction (PCR) product

RNA extraction, cDNA synthesis, the PCR and the quantification of the PCR product (QPCR) were performed as previously described (Swiderski et al., 1999). Briefly, for RNA extraction the rapidly thawed aliquots were processed to RNA by adding 300  $\mu\text{l}$  RNA Stat, an acid guanidium thiocyanate-phenol disassociation buffer (RNA stat-60, Tel Test, USA) and by performing the phenol: chloroform extraction procedure according to the manufacturer's instructions. RNA was quantified spectrophotometrically. For cDNA synthesis, 1.2  $\mu\text{g}$  RNA in 35  $\mu\text{l}$  diethyl pyrocarbonate (DEPC) water was heated for 10 min to denature the RNA and chilled on ice for 2 min. After adding 45  $\mu\text{l}$  of reagent master mix to the RNA solution, the final reaction volume of 80  $\mu\text{l}$  was left at room temperature for 10 min to facilitate primer annealing, incubated at  $40^\circ\text{C}$  for 1 h, diluted with 40  $\mu\text{l}$  DEPC water and finally frozen at  $-20^\circ\text{C}$  until used for PCR. For the PCR, the RT reaction was further diluted by 1:2 with

DEPC treated water. Target sequences were amplified in 50  $\mu\text{l}$  reactions containing Taq thermostable polymerase and primers based on the sequences for equine IFN- $\gamma$ , IL-4, IL-5, IL-13 and  $\beta$ -actin (housekeeping gene as internal control). The PCR reactions were performed in duplicate and the plasmid constituting the standard curve was placed on the same plate with the unknown RT reactions. For hybridisation and quantification of the PCR product, 5  $\mu\text{l}$  of PCR product were placed in a 50  $\mu\text{l}$  reaction containing 1  $\mu\text{l}$  target specific TBR labelled oligonucleotide probe, 3.5  $\mu\text{l}$  PCR Buffer without  $\text{MgCl}_2$  (Perkin-Elmer) and 45  $\mu\text{l}$  DEPC treated water. The reaction was heated to  $95^\circ\text{C}$  for 90 s followed by a 5 min incubation at  $55^\circ\text{C}$ . Biotinylated PCR product was captured by adding 15  $\mu\text{l}$  of streptavidin coated iron beads (Dynabeads, Perkin-Elmer) to the hybridisation reaction and incubating at  $5^\circ\text{C}$  for 30 min. The total 65  $\mu\text{l}$  reaction mixture was transferred to a polypropylene tube containing 335  $\mu\text{l}$  QPCR Assay Buffer (Perkin-Elmer) and quantified using the QPCR system 5000 (Perkin-Elmer). Copy numbers were corrected for  $\beta$ -actin content of RT reactions prior to analysis.

### 2.6. Statistical analysis

As data were not normally distributed, intra- and inter-group comparisons were made using Wilcoxon-Rank and Mann-Whitney tests, respectively.

## 3. Results

### 3.1. Clinical scores, airway function and BALF cytology

Hay/straw exposure significantly increased ( $p < 0.05$ ) clinical scores of RAO horses at 48 h (median score 2, range 2–8) when compared with baseline (median score 0, range 0–0). No significant increase in clinical score was detected in the control group (baseline score 0, 0–0; 48 h score 0, 0–1). There was no statistically significant alteration in respiratory mechanics or airway reactivity at 48 h in either group. While hay/straw challenge significantly increased absolute BALF neutrophil counts ( $p = 0.027$ ) and neutrophil ratios ( $p = 0.036$ ) in both groups, RAO horse had significantly higher post-challenge neutro-

Table 1

Absolute neutrophil and lymphocyte counts ( $\times 10^5/\text{ml}$ ) and neutrophil and lymphocyte ratios (%) in BALF from control ( $n = 5$ ) and RAO ( $n = 7$ ) horses at 0 and 48 h after hay/straw exposure

Time (h)	Control	RAO
Neutrophil ratio		
0	1.0 (0.4–2.9)	1.5 (0.6–4.2)
48	6.4 (3.0–11.6)	23.2 (12.7–49.7)
Absolute neutrophil count		
0	0.03 (0.01–0.07)	0.04 (0.01–0.19)
48	0.27 (0.08–0.41)	1.97 (0.32–4.33)
Lymphocyte ratio		
0	38.3 (31.1–62.0)	37.3 (18.6–53.4)
48	38.7 (26.2–56.0)	37.7 (27.5–49.9)
Absolute lymphocyte count		
0	0.06 (0.01–1.00)	0.05 (0.03–0.13)
48	0.41 (0.08–1.39)	1.43 (0.13–3.73)

phil counts ( $p = 0.027$ ) and ratios than controls ( $p < 0.0034$ ; Table 1). There was no significant difference in absolute PBL number in either group, neither before nor after hay/straw exposure (Table 2). Hay/straw challenge significantly increased absolute BALL numbers in RAO horses ( $p = 0.022$ ; Table 1). There were no significant intra- or inter-group differences in BALF macrophage and eosinophil numbers.

### 3.2. Separation of BALL and PBL subpopulations

BALL and PBL were successfully separated into CD4 and CD8 using magnetic bead selection, with the separated populations comprising median 91.2% (range 74–98%) BALL CD4 cells, median 94.7% (range 83–99%) BALL CD8 cells and 93.7% (range 62–100%) PBL CD4 and median 87.4% (range 62–99%) PBL CD8 cells, respectively.

### 3.3. Cytokine mRNA expression

Hay/straw challenge had no statistically significant effect on PBL or BALL cytokine mRNA expression in

Table 2

Absolute PBL counts ( $\times 10^6/\text{ml}$ ) for control ( $n = 5$ ) and RAO ( $n = 7$ ) horses at 0 and 48 h after hay/straw exposure

	Control	RAO
Absolute PBL count at 0 h	1.59 (0.69–2.82)	1.55 (0.90–2.88)
Absolute PBL count at 48 h	2.19 (1.07–4.50)	1.00 (0.64–2.02)

either group, although controls had an increase in IL-13 expression in CD8 PBL at 48 h that approached significance (0 h median 206,844 corrected copy numbers, range 92,706–262,098; 48 h median 728,294, range 138,482–1881,048;  $p = 0.059$ ; Fig. 1).

There were significant inter-group differences in PBL (Fig. 1) and BALL (Fig. 2) cytokine expression at 0 and 48 h. At baseline, compared with RAO horses, controls had significantly higher IL-13 (control 206,844, 92,706–262,098; RAO 6837, 2036–82,225;  $p = 0.0058$ ) and INF- $\gamma$  (control 1,034,772, 349,961–2,953,582; RAO 18,268, 3565–22,7369;  $p = 0.0058$ ) expression by CD8 PBL, significantly lower IL-13 expression by CD4 BALL (control 6326, 4754–12,254; RAO 25,084, 5658–68,6947;  $p = 0.035$ ), and an increase in INF- $\gamma$  expression by CD4 PBL that approached significance (control 143,275, 18,115–2,055,783; RAO 10,338, 4705–778,097;  $p = 0.051$ ).

At 48 h, compared with RAO horses, controls had significantly increased expression of IL-13 in CD4 PBL (controls 88,482, 579–223,050, RAO 8442, 3684–82,274;  $p = 0.015$ ), IL-13 (controls 728,294, 138,482–1,881,048; RAO 8681, 3282–263,886;  $p = 0.015$ ) and INF- $\gamma$  (controls 1,381,358, 604,278–2,497,126; RAO 8827, 6827–250,587;  $p = 0.0058$ ) expression in CD8 PBL, IL-5 expression in CD4 BALL (controls 306,082, 26,069–624,056, RAO 12,801, 2845–75,652;  $p = 0.023$ ), and an increase in expression of INF- $\gamma$  in CD4 PBL that approached significance (controls 244,483, 7522–271,7234, RAO 10,544, 4257–142,269;  $p = 0.051$ ).

## 4. Discussion

Hay/straw exposure induced clinical airway obstruction, airway neutrophilia and airway lymphocytosis in RAO horses, and, consistent with previous findings (McGorum et al., 1993), induced a mild, but significant, airway neutrophilia in controls. However, hay/straw exposure had no significant effect on PBL or BALL cytokine mRNA expression in either group. This is consistent with one previous study (Ainsworth et al., personal communication), but differs from other studies (Bowles et al., 2002; Lavoie et al., 2001) which suggested that RAO was associated with a predominantly Th2 response or a mixed Th1/Th2 response. These contradictory results likely reflect differences in

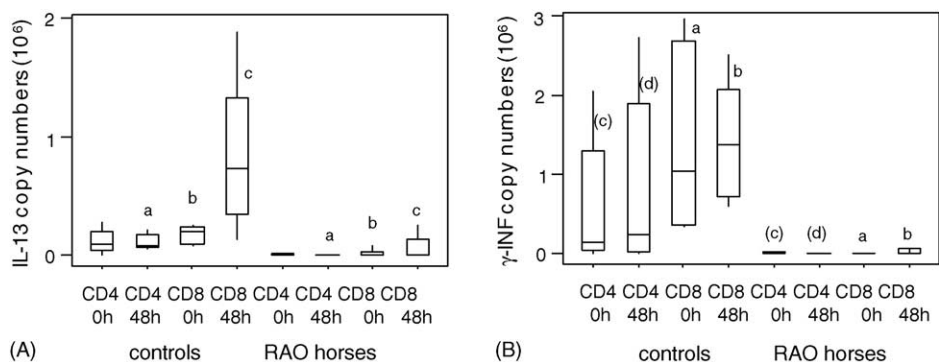


Fig. 1. IL-13 and  $\gamma$ -INF mRNA expression (copy numbers are normalised for  $\beta$ -actin content of RT reactions) of CD4 T and CD8 PBL from RAO ( $n = 7$ ) and control ( $n = 5$ ) horses at 0 and 48 h after hay/straw exposure. Lower case indicates significant inter-group differences ( $p < 0.05$ ). Lower case in brackets indicates inter-group difference that approaches significance ( $p < 0.06$ ).

disease phenotype and/or study protocol. The aim of the present study was the quantification of cytokine mRNA in specific lymphocyte subpopulations, rather than in mixed lymphocyte populations. Thus, in contrast to previous studies, this is the only study to quantify cytokine expression in purified CD4 and CD8 populations, thereby overcoming differences in cytokine expression which may have resulted from differences in proportions of lymphocyte populations or in the mixed cell populations present in BALF samples. The duration of hay/straw challenge prior to sampling is also likely to have had an important effect on cytokine expression. Indeed Cordeau et al. (2002) demonstrated that the development of a Th2 cytokine response coincided temporally with development of airway obstruction. In two studies (Giguere et al.,

2002; Lavoie et al., 2001), BALL cytokine expression was assessed when horses developed marked airway obstruction, while in the present study and in that of Ainsworth et al., it was assessed at earlier time points after hay/straw exposure (48 and 24 h, respectively), when airway obstruction was less severe. The differences in the cytokine expression among studies probably also reflect differences in the levels of exposure to the numerous pro-inflammatory agents present in hay/straw. For example, inhalation of *Aspergillus fumigatus* promotes a Th2 response (Yamashita et al., 2002), while inhalation of endotoxin promotes a Th1 response (Wesselius et al., 1997).

While hay/straw exposure had no effect on either PBL proportion, it significantly increased the proportion of CD8+ BALLs in RAO horses. Additionally the

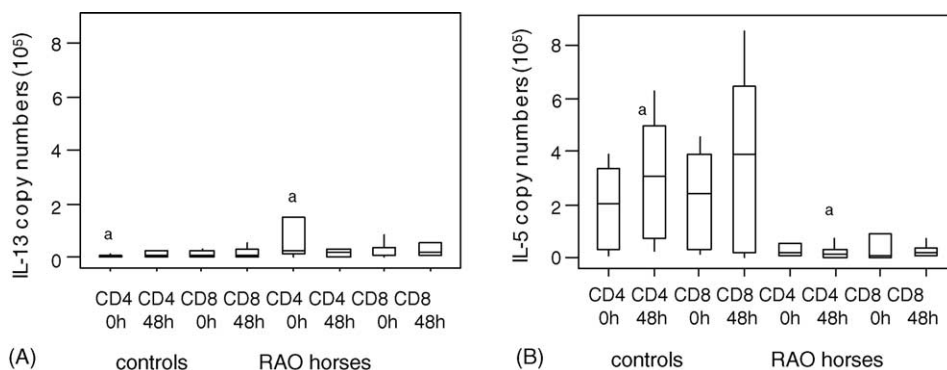


Fig. 2. IL-13 and  $\gamma$ -INF mRNA expression (copy numbers are normalised for  $\beta$ -actin content of RT reactions) of CD4 and CD8 BALL from RAO ( $n = 7$ ) and control ( $n = 5$ ) horses at 0 and 48 h after hay/straw exposure. Lower case indicates significant inter-group differences ( $p < 0.05$ ).

proportion of CD8+ BALLs at 48 h was significantly higher in RAO horses when compared to controls. A similar elevation of the percentage and number of CD8+ BALLs was previously found in RAO horses (Kleiber et al., 1999).

While hay/straw exposure had no significant effect on PBL phenotype distribution in either group, RAO horses had a significantly higher proportion of CD8+ PBL at 48 h. While the relevance of this observation is unclear, a similar increase in CD8+ PBL occurs in subjects with late asthmatic reactions to inhaled toluene diisocyanate (Finotto et al., 1991).

Interestingly, RAO and control horses differed with respect to baseline cytokine expression in PBL, with RAO horses having lower baseline expression of IL-13 and IFN- $\gamma$  in CD8 PBL, and a reduction in INF- $\gamma$  expression in CD4 PBL which approached significance. This suggests that RAO horses have a persistent defect in cytokine production, even during remission, when airway neutrophilia and dysfunction were absent. Consistent with this finding, bronchial epithelial cells from RAO horses had a cytokine (IL-1 $\beta$  and TNF- $\alpha$ ) mediated up-regulation of nuclear factor- $\kappa$ B which persisted for at least 21 days after cessation of antigen exposure (Bureau et al., 2000a, 2000b). A similar reduction in INF- $\gamma$  expression in PBL occurs in horses with active summer associated obstructive pulmonary disease (SPAOPD), another form of equine RAO, when compared with SPAOPD horses in remission and control horses (Beadle et al., 2002). Similarly, atopic children have defective INF- $\gamma$  expression in PBL at birth, prior to the onset of atopic disease (Smart and Kemp, 2002).

While hay/straw exposure had no significant effect on lymphocyte cytokine expression in either group, controls had an increase in expression of the Th2 cytokine IL-13 in CD8 PBL at 48 h that approached significance. While the biological significance of this is unclear, other significant inter-group differences in IL-13 expression were also noted. Compared with controls, RAO horses had a significantly lower baseline and post-challenge IL-13 expression in CD8 PBL, lower post-challenge expression of IL-13 in CD4 PBL, and higher baseline expression of IL-13 in CD4 BALL. The former two observations are consistent with the elevated expression of IL-13 noted in some SPAOPD horses when in disease remission (Bowles et al., 2002). The biological significance of

these alterations in expression of IL-13 is unclear, although IL-13 acts as a central regulator of the murine allergic response, being capable of inducing the entire allergic asthmatic phenotype independent of other Th2 cytokines (Wills-Karp and Chiaramonte, 2003; Wills-Karp et al., 1998). However it is interesting that the increases in IL-13 expression, for RAO and control horses, respectively, occurred in the airway (BALL) and systemic (PBL) compartments.

There was no detectable alteration in the expression of the Th2-type cytokine IL-4 in PBL and BALL, in either group, consistent with previous findings in RAO horses given acute (Giguere et al., 2002) and chronic (Giguere et al., 2002) hay/straw exposure. The relevance of the increased expression of the Th2-type cytokine IL-5 in CD4 BALL in controls at 48 h after hay/straw exposure is unclear. While IL-5 has a pivotal role in eosinophil trafficking (Hogan et al., 1998), none of the horses had airway eosinophilia post-challenge.

Interestingly, RAO horses appeared to have a general down-regulation in PBL and BALL mRNA cytokine expression. Indeed, except for the higher baseline IL-13 expression in CD4 BALL, for all of the significant inter-group differences in cytokine expression, expression was greater in controls. This is surprising since the hay/straw challenge induced a significantly greater inflammatory response in RAO horses, and given the prevailing notion that horses with RAO have exaggerated immune responses. While cytokine down-regulation occurs in mice following continued inhaled antigen exposure (Hoyne et al., 1996, 1993, 1997), this would seem unlikely to have occurred in this study since the RAO horses developed airway inflammation and dysfunction following hay/straw exposure. Lastly it should be noted that this and other studies of equine cytokine expression have relied upon the detection of cytokine-specific mRNA as surrogates for protein expression. While the underlying assumption of this approach is that cytokine expression is regulated at the level of mRNA transcription, the possibility exists for post-transcriptional regulation. Direct detection of cytokine protein expression may be necessary to confirm Th cytokine expression patterns in RAO-affected horses.

In summary, in this study, RAO was not associated with alterations in lymphocyte cytokine expression that are consistent with Th1 or Th2 responses, but

rather with a general down-regulation in expression of the measured cytokines in PBL and BALL.

## Acknowledgements

The study was funded by the Horserace Betting Levy Board, the Faculty of Veterinary Medicine, University of Edinburgh and the United States, Department of Agriculture.

## References

- Beadle, R.E., Horohov, D.W., Gaunt, S.D., 2002. Interleukin-4 and interferon-gamma gene expression in summer pasture-associated obstructive pulmonary disease affected horses. *Equine Vet. J.* 34, 389–394.
- Bowles, K.S., Beadle, R.E., Mouch, S., Pourciau, S.S., Littlefield-Chabaud, M.A., Le Blanc, C., Mistic, L., Fermaglich, D., Horohov, D.W., 2002. A novel model for equine recurrent airway obstruction. *Vet. Immunol. Immunopathol.* 87, 385–389.
- Brazil, T.J. (2000). Pulmonary neutrophil recruitment, activation and clearance in equine COPD. University of Edinburgh.
- Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., Merville, M.P., Bours, V., Lekeux, P., 2000a. Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am. J. Respir. Crit. Care Med.* 161, 1314–1321.
- Bureau, F., Delhalle, S., Bonizzi, G., Fievez, L., Dogne, S., Kirschvink, N., Vanderplasschen, A., Merville, M.P., Bours, V., Lekeux, P., 2000b. Mechanisms of persistent NF-kappa B activity in the bronchi of an animal model of asthma. *J. Immunol.* 165, 5822–5830.
- Cordeau, M.E., Joubert, P., Hamid, Q., Lavoie, J.P., 2002. Temporal mRNA expression of TH2-type cytokine in an animal model of chronic asthma. *Am. J. Respir. Crit. Care Med.* A424.
- Finotto, S., Fabbri, L.M., Rado, V., Mapp, C.E., Maestrelli, P., 1991. Increase in numbers of CD8 positive lymphocytes and eosinophils in peripheral blood of subjects with late asthmatic reactions induced by toluene diisocyanate. *Br. J. Ind. Med.* 48, 116–121.
- Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J., Franchini, M., 2002. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet. Immunol. Immunopathol.* 85, 147–158.
- Hogan, S.P., Mould, A.W., Young, J.M., Rothenberg, M.E., Ramsay, A.J., Matthaei, K., Young, I.G., Foster, P.S., 1998. Cellular and molecular regulation of eosinophil trafficking to the lung. *Immunol. Cell Biol.* 76, 454–460.
- Hoyne, G.F., Askonas, B.A., Hetzel, C., Thomas, W.R., Lamb, J.R., 1996. Regulation of house dust mite responses by intranasally administered peptide: transient activation of CD4+ T cells precedes the development of tolerance in vivo. *Int. Immunol.* 8, 335–342.
- Hoyne, G.F., Callow, M.G., Kuo, M.C., Thomas, W.R., 1993. Characterization of T-cell responses to the house dust mite allergen Der p II in mice. Evidence for major and cryptic epitopes. *Immunology* 78, 65–73.
- Hoyne, G.F., Jarnicki, A.G., Thomas, W.R., Lamb, J.R., 1997. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int. Immunol.* 9, 1165–1173.
- Kleiber, C., Grunig, G., Jungi, T., Schmucker, N., Gerber, H., Davis, W.C., Straub, R., 1999. Phenotypic analysis of bronchoalveolar lavage fluid lymphocytes in horses with chronic pulmonary disease. *Zentralbl. Veterinarmed A* 46, 177–184.
- Lavoie, J.P., Maghni, K., Desnoyers, M., Taha, R., Martin, J.G., Hamid, Q.A., 2001. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am. J. Respir. Crit. Care Med.* 164, 1410–1413.
- McGorum, B.C., Dixon, P.M., 1992. Preliminary observations on inhalation and intradermal challenges of horses with oil seed rape. *Vet. Rec.* 131, 163–167.
- McGorum, B.C., Dixon, P.M., Halliwell, R.E., 1993. Phenotypic analysis of peripheral blood and bronchoalveolar lavage fluid lymphocytes in control and chronic obstructive pulmonary disease affected horses, before and after 'natural (hay and straw) challenges'. *Vet. Immunol. Immunopathol.* 36, 207–222.
- Pirie, R.S., Collie, D.D., Dixon, P.M., McGorum, B.C., 2003. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin. Exp. Allergy* 33, 676–683.
- Pirie, R.S., Dixon, P.M., Collie, D.D., McGorum, B.C., 2001. Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet. J.* 33, 311–318.
- Smart, J.M., Kemp, A.S., 2002. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. *Clin. Exp. Allergy* 32, 796–802.
- Swiderski, C.E., Klei, T.R., Horohov, D.W., 1999. Quantitative measurement of equine cytokine mRNA expression by polymerase chain reaction using target-specific standard curves. *J. Immunol. Methods* 222, 155–169.
- Watson, J.L., Stott, J.L., Blanchard, M.T., Lavoie, J.P., Wilson, W.D., Gershwin, L.J., Wilson, D.W., 1997. Phenotypic characterization of lymphocyte subpopulations in horses affected with chronic obstructive pulmonary disease and in normal controls. *Vet. Pathol.* 34, 108–116.
- Wesseliuss, L.J., Nelson, M.E., Bailey, K., O'Brien-Ladner, A.R., 1997. Rapid lung cytokine accumulation and neutrophil recruitment after lipopolysaccharide inhalation by cigarette smokers and nonsmokers. *J. Lab. Clin. Med.* 129, 106–114.
- Wills-Karp, M., Chiamonte, M., 2003. Interleukin-13 in asthma. *Curr. Opin. Pulm. Med.* 9, 21–27.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258–2261.
- Yamashita, Y., Okano, M., Yoshino, T., Hattori, H., Yamamoto, T., Watanabe, T., Takishita, T., Akagi, T., Nishizaki, K., 2002. Carbohydrates expressed on *Aspergillus fumigatus* induce in vivo allergic Th2-type response. *Clin. Exp. Allergy* 32, 776–782.