Veterinary Immunology and Immunopathology 202 (2018) 70–73

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short Communications

Tumor necrosis factor-alpha protein concentrations in bronchoalveolar lavage fluid from healthy horses and horses with severe equine asthma

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ARTICLE INFO

Keywords:
Severe equine asthma
Neutrophilic lower airway inflammation
Tumor necrosis factor-alpha protein concentration

ABSTRACT

The objective of this study was to determine if TNF-α protein concentration differs in bronchoalveolar lavage fluid (BALF) obtained from healthy horses, horses with naturally occurring exacerbations of severe equine asthma and horses in remission from severe equine asthma. Tumor necrosis factor-alpha (TNF- α) protein concentrations were determined in BALF by commercial equine ELISA. Horses with naturally occurring exacerbation of severe equine asthma were found to have significantly lower BALF TNF-α protein concentrations than healthy horses ($p = 0.0026$). There was no significant difference in BALF TNF-α concentration between horses in exacerbation and remission from disease, but there was a decrease in median TNF-α concentration between healthy horses and horses with clinical exacerbation of severe equine asthma. These findings suggest, that similar to human asthma, the role of TNF-α in chronic lower airway inflammation of horses may differ between disease phenotypes and disease state. Furthermore, the method with which TNF-α is measured (mRNA expression vs. protein concentration) may affect results when studying the role of TNF-α in horses with severe equine asthma.

1. Introduction

Equine asthma is a chronic inflammatory disease of the lower airways of horses, associated with exposure to environmental airborne allergens. The equine asthma syndrome includes several disease phenotypes, including mild to moderate equine asthma (formerly known as inflammatory airway disease, IAD) and severe equine asthma (formerly known as recurrent airway obstruction, RAO or heaves) (Couetil et al., 2016).

Severe equine asthma is clinically characterized by a chronic cough, exercise intolerance, and increased respiratory effort at rest. Horses suffering from severe equine asthma usually have increased numbers of neutrophils (> 20%) upon examination of bronchoalveolar lavage fluid (BALF) cytology, whereas milder forms of asthma can also be eosinophilic or mast cells may be the predominant inflammatory cell type (Couetil et al., 2016). Similar to the human asthma syndrome, it has been proposed that such differences in inflammatory cell type are the result of different pathophysiological pathways in horses with asthma (Lavoie et al., 2011). It can be assumed that the dominating inflammatory cell type (i.e. neutrophils, eosinophils, mast cells) should be the result of specific signalling to induce inflammatory cell recruitment, establishment and persistence of inflammation.

Even though the immunopathogenesis of severe equine asthma has not been fully determined, the potential role of several cytokines in neutrophil recruitment and establishment of airway inflammation has been studied. One of the commonly studied pro-inflammatory cytokines is tumor necrosis factor alpha (TNF-α), which has a role in neutrophil activation and an increase in TNF-α mRNA expression has previously been found in horses with experimental exacerbation of severe equine asthma (Giguère et al., 2002; Padoan et al., 2013). However, measuring gene expression does not take posttranscriptional events into account (Lavoie-Lamoureux et al., 2010; Richard et al., 2014) and may not be representative of biologically active TNF-α. The objective of this study was to determine if TNF-α protein concentrations differ in BALF obtained from healthy horses, horses with naturally occurring exacerbations of severe equine asthma and horses in remission from severe equine asthma. Our hypothesis was that horses with clinical equine asthma have higher BALF TNF-α protein concentration when compared

Abbreviations: BALF, bronchoalveolar lavage fluid; IAD, inflammatory airway disease; RAO, recurrent airway obstruction; TNF-α, tumor necrosis factor-alpha

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https://doi.org/10.1016/j.vetimm.2018.06.014
Received 3 April 2018; Received in revised form 12 June 2018; Accepted 23 June 2018

to healthy horses and those in remission.

2. Materials and methods

2.1. Study population

Banked samples from other equine asthma research projects of the first author were used for analysis. Samples had been collected from client owned horses on their respective home farm in the summer months (May– September) in Saskatchewan, Western Canada. Horses were either experiencing a clinical exacerbation of severe equine asthma (exercise intolerance, mucopurulent nasal discharge, cough, increased respiratory effort at rest), had a history of being previously diagnosed with severe equine asthma by a veterinarian with absence of clinical signs at the time of examination (remission), or were considered systemically healthy with no previous history of lower airway disease. None of the horses had received treatments for equine asthma for at least two months prior to sample collection. The studies had been approved by the University of Saskatchewan Institutional Animal Care and Use Committee. Owners of the horses provided written consent for clinical examination and sample collection procedures.

2.2. Clinical examination and scoring

A distant examination was performed and a clinical score was assigned with a 1–4 scale for abdominal effort and for nostril flaring at rest, respectively (Robinson et al., 2000). The scores were then combined, with an overall score of 2 being normal and 8 defined as severe respiratory distress. The amount of mucus present within the trachea was scored during airway endoscopy on a 0–5 scale ranging from no mucus to profuse amounts (Gerber et al., 2004). Horses also underwent a physical examination, including rebreathing examination, as part of their enrolment (JM or MH). A complete blood count (CBC) was performed to help rule out signs of systemic inflammation (data not provided). Horses with an abnormal CBC were excluded from the study.

2.3. Sample collection

Bronchalveolar lavage (BAL) was performed in horses under standing sedation (detomidine and butorphanol) using a blind technique with a BAL catheter (Mila International, Inc.). The catheter was passed from the nostril, into the trachea, and wedged into a 1 cm diameter bronchus where the cuff was inflated. Airway mucosal anesthesia was induced by instilling 20–30 ml of 2% lidocaine through the catheter during placement to reduce coughing. Three aliquots of 120 ml of sterile saline were infused and aspirated consecutively, through the catheter. The first aliquot was discarded and the second and third samples were pooled and immediately placed on ice (Jean et al., 2011). Samples were submitted to Prairie Diagnostic Services, Inc. (Saskatoon, SK, Canada) for cytological analysis as part of two other ongoing research projects. The remaining fluid was centrifuged and the supernatant was removed and frozen in 1 ml aliquots at −80 °C.

2.4. Cytological analysis

BAL fluid (BALF) was assessed for its appearance, and direct and cytocentrifuge preparations (Cytospin 2) were prepared and stained with a Romanowsky stain (Hema 3 Stain Set). Slides were examined by one of two board certified clinical pathologists (HB and MM), using the same protocol. A 200 cell differential count was performed on the cytocentrifuge preparation, on 100x magnification with immersion oil, as is standard protocol for the diagnostic lab (Prairie Diagnostic Services, Inc.). Slides were also assessed for cellularity, cellular preservation, mucus content, degree of cellular entrapment, the presence of respiratory epithelium, Curschmann's spirals, environmental contaminants, and etiologic agents.

2.5. Tumor necrosis factor-alpha (TNF-α) ELISA

Following collection of samples from all horses, BALF supernatant was thawed at room temperature for use in a commercially available enzyme-linked immunosorbent assay (ELISA) for equine TNF-α (R&D Systems DuoSet ELISA Equine TNF-α). The assay was performed according to manufacturers' instructions and repeated twice in quadruplicate. The quantification range for TNF-α was 3.6–500 pg/mL. Briefly, for the TNF-α ELISA, blank 96 well microplates were coated with equine TNF-α capture antibody, and samples or standard were added followed by equine TNF-α detection antibody. Streptavidin-HRP was then added to each well followed by a substrate solution and a stop solution. The optical density was determined with a microplate reader set to 450 nm. Transforming the wavelengths of the ELISA standards into a log scale and graphing the sample results along this scale determined results.

2.6. Statistical analysis

Data was analyzed with a commercial software program (GraphPad Prism 6). Data was tested for normality and a nonparametric Kruskal-Wallis test, with Dunn's multiple comparisons test, was used for not normally distributed and ranked (clinical and tracheal mucus score) data. A P-value of ≤ 0.05 was considered significant.

3. Results and discussion

Study population and clinical data are summarized in Table 1. Samples from 44 horses were included in the analysis, including seven healthy horses, six horses in asthma remission and 31 horses with asthma exacerbation. Breeds were representative of the horse population in our region, including Quarter Horse (18), Paint (10), Appaloosa (6), Arabian (3), Clydesdale (2), and one each of Morgan, Warmblood, Standardbred, Thoroughbred, and Pony of the Americas. Age of horses ranged from four to 23, with no difference in age between the groups ($p = 0.2347$; Table 1).

Clinical score was different in horses with asthma exacerbation when compared to healthy horses and those in remission ($p < 0.0001$; Fig. 1). One horse included in the exacerbation group had normal clinical examination findings on the day of sample collection, but had shown clinical signs consistent with acute asthma exacerbation the previous day. There was no difference in tracheal mucus score between horses in remission from asthma and those with asthma exacerbation; however, both asthma groups were different from healthy horses ($p = 0.0035$; Fig. 1). Percent BALF neutrophils was different between healthy horses and horses with asthma exacerbation with no difference observed between horses in asthma remission with either group ($p < 0.0001$; Fig. 2).

The concentration of TNF-α in BALF was different between healthy

Table 1
Age, gender and clinical data from healthy horses, horses in remission from severe equine asthma and horses with naturally occurring severe equine asthma exacerbation. Data presented include the range (25^{th};75^{th} percentile). Results with different superscripts are significantly different from each other. F = female, MC = male castrated, M = male; BALF = bronchoalveolar lavage fluid.

Horses (n = 44)	Age (years)	Gender (n)	Clinical score	Tracheal mucus score	% BALF neutrophils
Healthy (n = 7)	7-20 (7;13)	F (5) MC (2)	2[a] (2;2)	0-2[a] (0;2)	0-5[a] (1;2.5)
Remission (n = 6)	8-20 (9.5;19.3)	F (5) MC (1)	2-3[a] (2;2.3)	2-4[b] (2.8;4)	4-23.5[ab] (4;19.4)
Exacerbation (n = 31)	4-23 (9.8; 20)	F (13) M (1) MC (17)	2-8[b] (4;6)	1-4.5[b] (1;3.5)	6-81[b] (14;45)