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Research paper

Cytokine mRNA expression of pulmonary macrophages varies with challenge but not with disease state in horses with heaves or in controls

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ABSTRACT

Heaves in horses is characterized by lower airway neutrophilic inflammation, and reversible airflow obstruction. Pulmonary macrophages contribute to the inflammation observed in a number of human and animal pulmonary diseases, and it has been postulated that they are responsible for the neutrophilic inflammation present in heaves by the release of cytokines and chemokines. To test this hypothesis, the mRNA expression of TNF-α, IL-1β, IL-8, and MIP-2 by macrophages isolated from bronchoalveolar lavage cells was quantified using real-time RT-PCR in horses with heaves (n-6) and controls (n-6). Animals were studied after being pastured for 3 months to induce clinical remission of heaves, and after 24 h, and 9 days of a continuous natural antigen challenge consisting of hay feeding and straw bedding. The study was performed during 2 consecutive summers, when 3 horses with heaves and 3 control horses were evaluated. As expected, airway obstruction developed with the challenge only in horses with heaves, while airway neutrophilia was observed in both groups of horses. Stabling resulted in an increased expression of IL-8/ß-actin and MIP-2/ß-actin after 24 h of stabling in both groups of horses. Further analyses revealed that compared to pasture, the expression of these chemokines was significantly increased after 24 h of stabling only in Year 1, while the IL-8 expression was significantly decreased at 9 days in Year 2. No significant group, time, or year differences in IL-1β/ß-actin and TNF-α/ß-actin ratio were observed. The expression of IL-1β was strongly correlated with neutrophil percentages, although at different time points in the two study-years. These results suggest that alveolar macrophages can contribute to the airway inflammation resulting from stabling in horses by the release of IL-8 and MIP-2, but that the release of these chemokines is unlikely to be responsible for the marked airway neutrophilia observed in heaves. The variable expression of IL-8 and MIP-2 by alveolar macrophages between the two-study years are additional novel findings highlighting the complexity of the inflammatory pathways associated with airway inflammation and the importance of evaluating concurrently horses with heaves and controls to ensure identical environmental challenges.

1. Introduction

Equine heaves, also called recurrent airway obstruction (RAO), is characterized by lower airway inflammation, airway hyperresponsiveness and reversible airway obstruction when susceptible horses are stabled and exposed to hay feeding and straw bedding. The inflammatory response is associated with an accumulation of neutrophils within

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the airway lumen, and primarily mononuclear cells within the airway wall (Robinson, 2001; van der Haegen et al., 2005). However, the cells and immune pathways responsible for the development of airway inflammation and obstruction in heaves remain poorly understood.

It has been suggested that alveolar macrophages contribute to the airway inflammation in heaves as they are the most abundant phagocytes in healthy airways, and thus may be the first to encounter the offending antigens (Franchini et al., 1998). In support for a role of alveolar macrophages in heaves, *ex vivo* experiments have shown that they may produce pro-inflammatory cytokines and chemokines in response to antigens (Laan et al., 2006). C-X-C chemokines such as IL-8 (CXCL-8) and MIP-2 are central for neutrophils extravasation and migration in a large number of disease processes (Kobayashi, 2008). In the human and mouse airway lumen, they are produced primarily by resident cells, including structural cells and alveolar macrophages, in response to infectious agents and various other stimuli (Driscoll, 1994; Mukaida et al., 1998). In turn, the newly recruited neutrophils amplify the airway inflammatory response, by releasing pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-8 and MIP-2, and also reactive oxygen species, proteases, lipid mediators, microbicidal products, and nitric oxide (Cassatella, 1999; Gernez et al., 2010; Joubert et al., 2001).

It has previously been shown that IL-8, MIP-2, TNF-α and IL-1β are upregulated in BAL fluid cells of horses with heaves (Franchini et al., 2000; Franchini et al., 1998; Giguere et al., 2002). While both IL-8 and MIP-2 are potent chemoattractant for neutrophils, IL-8 also induces degranulation, attachment and trans-endothelial migration of neutrophils (Huber et al., 1991), and is also angiogenic (Koch et al., 1992). Inflammatory and immune signals including TNF-α and IL-1β are important inducer of C-X-C chemokines and their receptors is present on leucocytes and structural cells (Gernez et al., 2010; Govindaraju et al., 2006).

To our knowledge, the cytokine mRNA expression by equine alveolar macrophages before and following antigen challenges has not been previously evaluated in horses with heaves. Thus, we postulated that the mRNA expression of chemokines and pro-inflammatory cytokines by pulmonary macrophages is increased in the acute and chronic phases of the disease.

2. Materials and methods

2.1. Animals

Horses with heaves ($n = 6$, 14 ± 5.7 years, 475 ± 55 kg) had a well documented history of chronic respiratory disease and abnormal respiratory mechanics following exposure to moldy hay. Control horses ($n = 6$, 8 ± 2.6 years, 523 ± 63 kg) had no history or clinical signs of respiratory diseases when kept in the same environment. In Years 1 and 2, three horses with heaves and three control horses were pastured during the summer months for at least 3 months prior to the stabled together, exposed to moldy hay and bedded on straw. Different horses were studied in Years 1 and 2.

2.2. Lung function

Pulmonary mechanics parameters were obtained as previously described (Lavoie et al., 2006). In brief, flow rates were obtained by the use of a heated pneumotachograph and associated differential pressure transducer fitted to a mask placed over the horse's nose. Transpulmonary pressure (P_L) was obtained by subtracting the esophageal pressure from the mask pressure. Values of pulmonary resistance (R_L) were obtained by applying the data to the multiple regression equation for the single compartment model of the lung: $P_L = (E_L \times V) + (R_L \times \dot{V}) + K$ where E_L is the pulmonary elastance, V is the volume, $\dot{V}$ is the airflow and K is the transpulmonary end-expiratory pressure. The signals were sampled at a frequency of 120 Hz for 100 s and all valid breaths were used for analysis.

2.3. Bronchoalveolar lavage

Bronchoalveolar lavage were performed under sedation using a fibroptic endoscope, as previously described (Lavoie et al., 2006). The BAL fluid was collected in siliconized glass vessels and kept on ice. Total nucleated cells were counted using a hemocytometer. Smears of the fluid were prepared by centrifugation (at $90 \times g$ for 5 min) and stained with a modified Wright's solution. Differential counts were made on at least 400 cells, and did not include epithelial cells.

2.4. Cell isolation and viability

Macrophages were isolated using magnetic antibody cell separation (MACS) and a method modified from that described by Zahler et al. (1997). Briefly, BAL was centrifuged at $500 \times g$ for 5 min and the cell pellet was washed once with degassed phosphate buffered saline (PBS) supplemented with 0.5% low endotoxin fetal bovine serum (FBS) and 2 mM EDTA (PBS-buffer). The cells were incubated for 5 min on ice with a monoclonal antibody directed against equine macrophages (anti-macrophages CZ 3.3 WS II, kindly provided by Dr. Antczak, Cornell University, Ithaca, NY), centrifuged at $500 \times g$ and the cell pellet was resuspended with degassed PBS-buffer. Cells were centrifuged and rinsed two more times with 1 ml of degassed PBS-buffer and then incubated with an anti-mouse IgM antibody conjugated with microbeads for 5 min on ice, centrifuged, and resuspended in 3 ml of degassed PBS-buffer. A separation column was mounted on a magnetic support and washed at room temperature with 1 ml of degassed PBS-Buffer. The magnetically labeled cells were then loaded onto the column. The column was rinsed three times with 1 ml of degassed PBS-buffer, then removed from the magnetic field and flushed with 3 ml of PBS-buffer. Immediately after isolation, macrophages were suspended in culture medium at a concentration of 5×10^6 cells/ml and supplemented with 10% low endotoxin FBS, 2 mM of L-glutamine, 100 U/ml of penicillin and 100 ug/ml of streptomycin. Total cell counts were determined using a hemacytometer and viability was assessed by the Trypan blue dye exclusion test.