

Increased apoptosis of CD4 and CD8 T lymphocytes in the airways of horses with recurrent airway obstruction

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Abstract Recurrent airway obstruction (RAO, also known as equine heaves) is an inflammatory condition similar to human asthma caused by exposure of susceptible horses to poorly ventilated stable environments. The disease is characterized by neutrophilic airway inflammation, mucus hypersecretion and reversible bronchoconstriction. This inflammatory process is mediated by several factors, including antibodies, cytokines, resident cells of the airway and inflammatory cellular components that arrive in the respiratory tract. An increasing body of evidence has lent support to the concept that a dysregulation of T cell apoptosis may play a central role in the development of airway inflammation and the associated asthma. Therefore, the aim of this study was to investigate early and late apoptosis of CD4 and CD8 T cell subpopulations obtained from the airways of acute RAO-positive animals after exposure to hay/straw. The percentages of CD4 and CD8 T cells and their associated frequencies of apoptosis were quantified using flow cytometry. Hay/straw exposure induced clinical airway obstruction, airway neutrophilia and increased airway mucus production in RAO-positive horses. In addition,

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allergen exposure increased the percentage of CD4 T cells in RAO-positive horses as well as the frequency of early and late apoptosis in both CD4 and CD8 lymphocyte subpopulations. These results suggest that the higher frequency of lymphocyte apoptosis may play a role in disease progression of horses afflicted with RAO and may partially explain the characteristic remission of this pathological condition once the allergen source is removed. However, further studies are needed to clarify the role of T cell apoptosis in RAO-affected horses.

Keywords Apoptosis · T cells · Flow cytometry · Recurrent Airway Obstruction (RAO) · Horses

Abbreviations

BALF	bronchoalveolar lavage fluid
BALL	bronchoalveolar lavage lymphocytes
PBS	phosphate buffer solution
RAO	recurrent airway obstruction
7-AAD	7-aminoactinomycin D

Introduction

Recurrent airway obstruction (RAO, “heaves”) is an asthma-like condition that develops in mature horses following stabling and exposure to dusty hay and straw (Robinson 2001). Affected horses respond to this exposure by developing airway bronchoconstriction, neutrophilic inflammation and airway hyper-responsiveness. RAO is characterized by pulmonary neutrophilia and excessive mucous production, resulting in reduced dynamic lung compliance, increased pulmonary resistance and pleural pressure excursions (Derksen et al. 1985; Jackson et al. 2000).

It is commonly accepted that RAO disease is a bronchial hypersensitivity reaction induced by inhaled molds and organic dust (Derksen et al. 1988; McGorum et al. 1993a, b). However, although the immunological aspects of RAO have been extensively studied, the precise sequence of events is still not well understood (Leguillette 2003; Moran and Folch 2011). In general, airway inflammation involves the activation of pathogenic inflammatory cells, modulation of gene transcription factors and release of inflammatory mediators (Bureau et al. 2000a, b). Type I hypersensitivity, which is IgE-mediated (Halliwell et al. 1993; Schmallenbach et al. 1998; Eder et al. 2000; Eder et al. 2001; Curik et al. 2003; Künzle et al. 2007; Tahon et al. 2009; Moran et al. 2010a,b), and type III hypersensitivity reactions have been suggested to play roles in airway inflammation (Franchini et al. 2000; Lavoie et al. 2001).

T cells are important modulators of the immune response that are responsible for the pathogenesis of RAO (Robinson 2001; Moran and Folch 2011). The mechanism used by T cells to cause pathogenicity is likely through secretion of pro-inflammatory Th1 and Th2 cytokines induced by the humoral immune system (Ainsworth et al. 2003; Cordeau et al. 2004; Giguere et al. 2002; Lavoie et al. 2001; Riihimäki et al. 2008). Previous data has shown that RAO-affected horses produce these two types of apparently antagonistic cytokines depending on the stage of their disease and the timing of sample collection (Horohov et al. 2005). Through the use of quantitative PCR, researchers have demonstrated that bronchoalveolar lavage fluid (BALF) cells recovered from horses with heaves have increased expression of IFN- γ mRNA but no change in IL-4 or IL-13 mRNA levels (Giguere et al. 2002; Ainsworth et al. 2003; Horohov et al. 2005). Studies using *in situ* hybridization found a significant increase in the number of lung lymphocytes expressing IL-4 and IL-5 mRNA in RAO-affected horses 24 h after challenge in a dusty environment. These changes were

intensified after 9 days, at which time the number of cells positive for IFN- γ mRNA had decreased (Cordeau et al. 2004). In addition, it has also previously been shown that cytokine expression in airway lymphocytes is also influenced by the length of time that the RAO-affected horse has experienced clinical disease (Pietra et al. 2007).

Apoptosis is generally defined as a genetic program that eliminates unneeded, senescent, or damaged cells (Thompson 1995). Moreover, apoptosis is an important regulatory mechanism in the selection and containment of an immunocompetent T cell population, in T cell development and during immune responses. Dysregulation of apoptosis has been implicated in a range of diseases including tumors, neurodegenerative disorders, autoimmunity (Cohen 1999) and, perhaps, allergic asthma (Vignola et al. 1999; Woolley et al. 1996). Studies in human patients have demonstrated that reduced T cell apoptosis plays an important role in the pathogenesis of allergic bronchial asthma (Cormican et al. 2001; Vignola et al. 1999; De Rose et al. 2004). These findings are also consistent in murine models of asthma (Jayaraman et al. 1999; Tong et al. 2006; Finotto et al. 2007). In addition, increasing lines of evidence suggest that changes in programmed cell death mechanisms in both mobile and resident cells of the airway may directly contribute to the development and clinical severity of asthma (Vignola et al. 2000).

Given the link between apoptosis and asthma as well as the important role that lymphocytes play in the initial recruitment and activation of inflammatory cells and maintenance of disease in RAO-affected horses, the present work aimed to study the apoptotic status of CD4 and CD8 lymphocytes in normal and acute RAO-affected horses.

Materials and methods

Horses

Ten adult female horses were used in this study. Five horses with heaves (ages ranging from 4 to 20 years; body weights ranging from 420 to 450 kg) had a history of chronic respiratory disease following exposure to moldy hay (RAO herd). Five healthy control adult horses (ages ranging from 19 to 28 years; body weights ranging from 420 to 450 kg) with no detectable respiratory disease and without history and diagnosis of RAO were also studied.

All experimental procedures were approved by Institutional Animal Care and use Committee at Virginia Tech and by the Bioethics Committee for the Use of Animals in Biomedical Research of the Universidad Austral de Chile.

Hay/straw challenge

For the hay/straw challenge, horses were housed for 4 days in a poorly ventilated stable and fed with a mixture of good quality hay and hay with visible mold growth. This environment has previously been shown to induce airway inflammation and dysfunction in RAO-affected horses (McGorum et al. 1993a). The responses to the challenges was determined using clinical scoring, endoscopic examination and BALF cytology. Prior to the study, all horses were kept in a remission environment.

Clinical evaluation

Complete physical examinations and clinical scores were performed twice a day for each horse and included evaluations of rectal temperature, heart rate, respiratory rate, hydration,

gut motility, and auscultations of heart and lung. Clinical RAO scores were assigned as previously described by Robinson et al. (2000).

Endoscopic examination

Horses were restrained in stocks and sedated by intravenous administration of 0.01 mg/kg butorphanol (Fort Dodge, IA) and 0.01 mg/kg detomidine (Pfizer Exton, PA). A 3-m long endoscope was passed through the nasal passages and into the trachea until it wedged in a fourth-generation airway. The amount of mucus visible in the trachea was graded on a scale of 0 to 5, as previously described by Gerber et al. (2004).

Bronchoalveolar lavage fluid (BALF) collection and cytology

The endoscope was advanced until wedged, air was extracted by aspiration with a syringe, and three 100-ml aliquots of 37°C sterile saline solution were infused and re-aspirated. The aspirated fluid samples were mixed and pooled in a sterile specimen cup placed on ice. After collection, BALF samples were centrifuged at 200 x g for 15 min at 4°C. Total nucleated cell counts were determined from the cellular pellet using a hemacytometer. Differential cell counts were evaluated in a cytospin slide stained with a modified Wright's stain.

Preparation of BALF cells

Recovered BALF cells were placed in 30 ml of phosphate buffered saline (PBS) and centrifuged at 600 x g for 15 min. The supernatant was discarded after each spin cycle. After the second cell wash, the cells were resuspended in 2 ml of PBS. An aliquot of this suspension was taken, and living cells were counted using trypan blue staining and subsequently adjusted to a concentration of 1×10^6 cells/ml.

Identification of CD4 and CD8 T lymphocytes in BALF by flow cytometry

Fluorescent staining of BALF lymphocytes (BALL) was performed as follows. Prior to staining BALF cells, anti-horse CD4 and CD8 mouse IgG1 antibodies (VMRD, Inc, USA) were labeled using the Zenon® mouse IgG kit. Anti-CD4 was labeled with an anti-mouse IgG Fab conjugated to Alexa Fluor 647, and anti-CD8 was labeled with an anti-mouse IgG Fab conjugated to Alexa 488 using labeling kits (Invitrogen, USA) according to the manufacturer's instructions. Staining antibodies were diluted in buffer, added to cells, incubated for 30 min in the dark at 4°C and washed. Data were collected using a FACS Aria with three lasers (405 nm, violet; 488 nm, blue; and 633 nm, red) and analyzed using FlowJo (Treestar, Inc.).

Apoptosis determination in BALF lymphocytes

Early and late apoptotic lymphocytes were quantified by flow cytometry using a commercial Annexin-V Pacific Blue kit (BD Biosciences) according to the manufacturer's instructions. Cells were briefly suspended in Annexin-binding buffer at a concentration of 1×10^6 cells/ml. From this suspension, 100- μ l aliquots were dispensed into tubes, and 5 μ l of Annexin V-Pacific Blue (0.5 μ g/ml) and 10 μ l of 7-aminoactinomycin D (7-AAD) were added. The tubes were shaken and incubated in the dark for 15 min at room temperature. After incubation, 400 μ l of Annexin-binding buffer was added, and the samples were analyzed immediately by flow cytometry.

Statistical analysis

Differences between groups were determined using an unpaired Student's two-tailed *t*-test. Graph generation and statistical analyses were performed using Prism software (version 5.0; GraphPad).

Results

Clinical score, mucus score and BALF neutrophils in RAO-positive horses and normal controls

Hay/straw exposure significantly increased the clinical score of RAO-affected horses compared with normal controls ($P=0.029$). After 4 days of hay/straw exposure, RAO-affected horses exhibited a mean clinical score of 4 (range 4–6), while control horses had a mean clinical score of 2 (range 0–2). Mucus scores were also significantly increased in RAO-affected horses compared with normal animals ($P=0.002$); the mean score for RAO-positive animals was 2 with a range of 1.5–2.5, while the mean score for normal animals was 0. In addition, hay/straw challenge significantly increased the absolute numbers of BALF neutrophils in RAO-affected horses compared with the control group ($P=0.001$).

Quantification of BALL subpopulations in RAO-positive horses and normal controls

Hay/straw exposure had no statistically significant effect on the percentage of BALL within total BALF cells when control and RAO-affected horses were compared. In control horses, the mean percentage of BALL was 37.9% (range 35%–41.6%), and in RAO-positive horses, the mean was 38.63% (range 25.18%–54.98%). CD4 and CD8 lymphocytes were easily distinguished from total BALL using flow cytometry. Hay/straw exposure significantly increased ($P=0.001$) the percentage of CD4 cells in RAO-affected horses after 4 days of antigen exposure (mean 41.87%, range 38.17–44.25%) when compared with the control group (mean 24.76%, range 23.34–30.58%). However, there were no detectable changes in the CD8 population in either group after hay/straw exposure.

Quantification of early and late apoptosis in CD4 and CD8 BALL obtained from RAO-positive horses and normal controls

The frequencies of early and late apoptosis in CD4 cells of the RAO-positive animals after hay/straw exposure were significantly higher compared with those of the healthy controls (Fig. 1; $P=0.005$ and 0.002 , respectively). In the RAO-affected horses, the mean frequencies of early and late apoptosis were 29.36% and 26.39%, respectively, whereas in the healthy horses, the mean frequencies of early and late apoptosis were 12.21% and 7.6%, respectively. Similar results were obtained for CD8 cells. The frequencies of both early and late apoptosis in the CD8 cells of the RAO-affected horses were significantly higher compared with that of normal horses (Fig. 2; $P=0.009$ and 0.01 , respectively). In the RAO-positive horses, the mean frequencies of early and late apoptosis in CD8 cells were 35.75% and 20.46%, respectively, while the mean frequencies of apoptosis in the control horses were 9.79% of CD8 cells in early apoptosis, and 7.76% in late apoptosis.

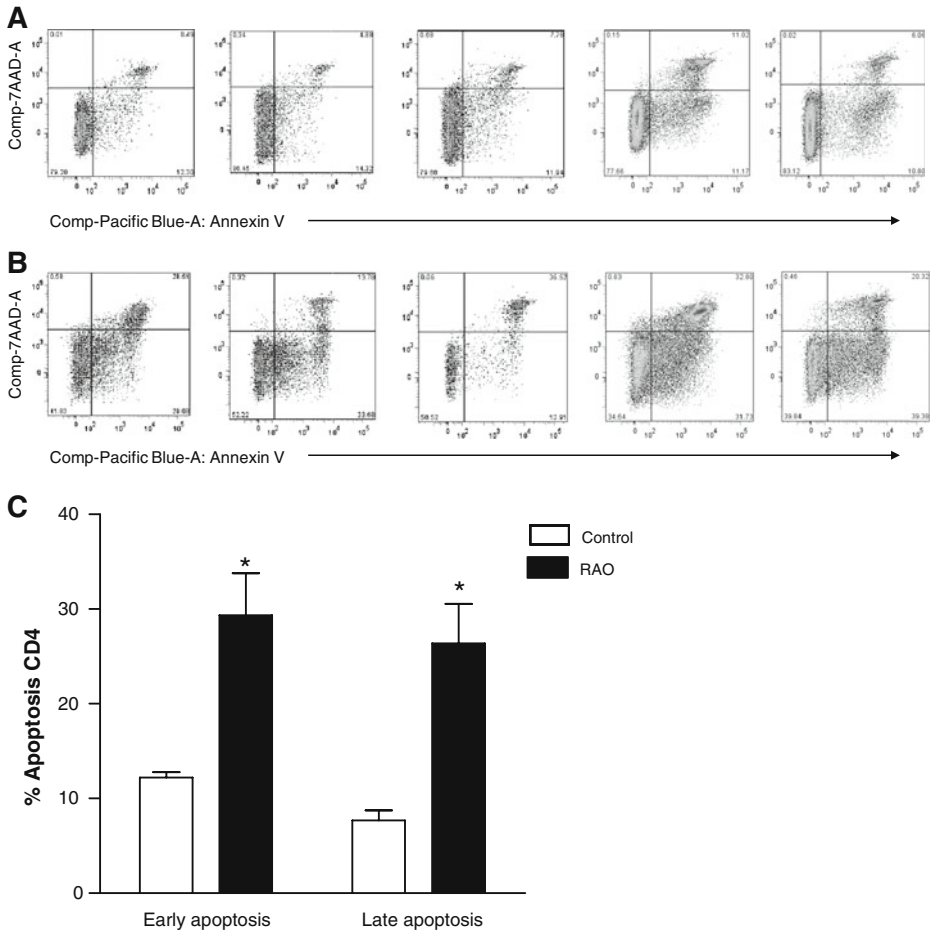


Fig. 1 Quantification of early and late apoptosis by flow cytometry. **a** Identification of early and late apoptosis in CD4 T cells in normal control horses. **b** Identification of early and late apoptosis in CD4 T cells in RAO-affected horses. **c** Percentage of early and late apoptosis in normal control horses and RAO positive horses. Each bar represents the mean of the apoptosis percentage, which was obtained from each group of horses (* $P < 0.01$ comparing normal control to RAO-affected horses)

Discussion

In the present study, consistent with previous studies, we found that hay/straw exposure induced clinical airway obstruction, airway neutrophilia, increased mucus score, and an increased proportion of CD4 cells in RAO-affected horses (McGorum et al. 1993b; Watson et al. 1997; Kleiber et al. 1999). We did not observe any changes in the CD8 subpopulation, as was also reported by other authors (McGorum et al. 1993b). However, other authors found that the proportion of CD8 BALLs was significantly higher in RAO horses when compared to controls (Kleiber et al. 1999; Kleiber et al. 2005). This finding raises the possibility that not only the CD4 cells but also the CD8 cells are involved in the pathogenesis of RAO.

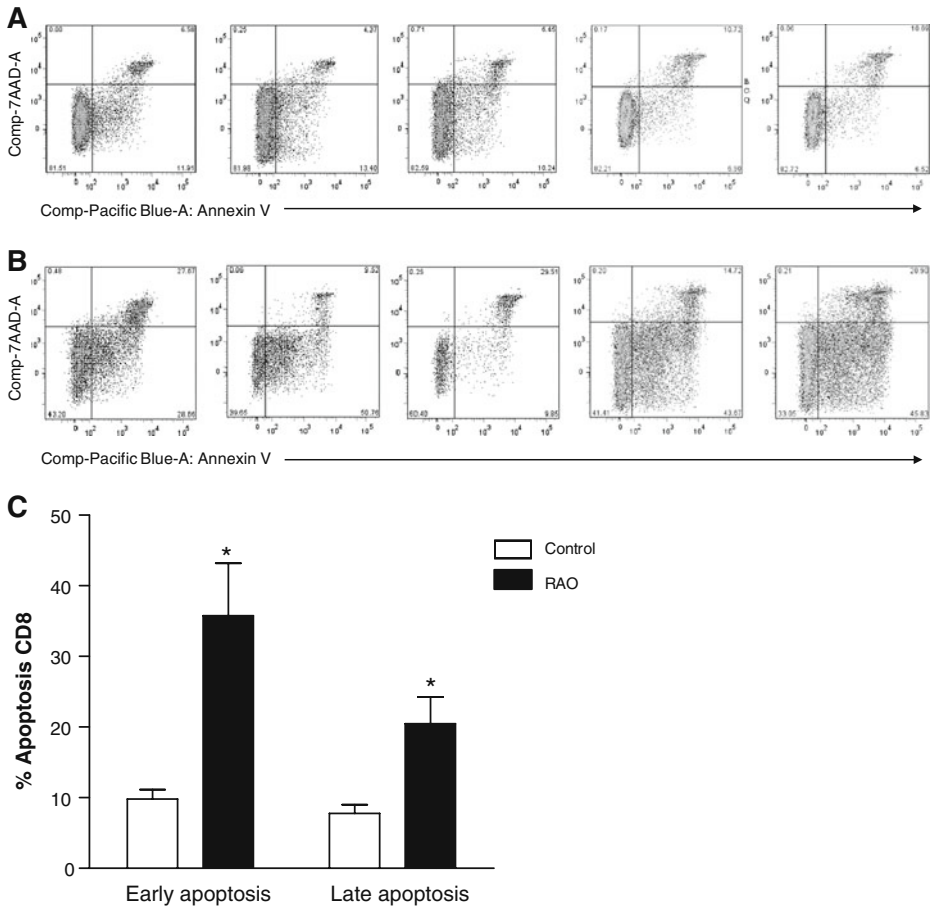


Fig. 2 Quantification of early and late apoptosis by flow cytometry. **a** Identification of early and late apoptosis in CD8 T cells in normal control horses. **b** Identification of early and late apoptosis in CD8 T cells in RAO-affected horses. **c** Percentage of early and late apoptosis in normal control horses and RAO positive horses. Each bar represents the mean of the apoptosis percentage, which was obtained from each group of horses (* $P < 0.05$ comparing normal control to RAO-affected horses)

We also unexpectedly found an increase in ongoing apoptosis of lymphoid cells recovered from the airways of RAO-affected horses. This result is in contradiction to the low frequencies of lymphocytes undergoing apoptosis reported in human asthma patient samples and experimental murine models. This finding could be explained by the presence of Th1 cytokines secreted in RAO-affected horses (Ainsworth et al. 2003; Cordeau et al. 2004; Giguere et al. 2002; Lavoie et al. 2001; Riihimäki et al. 2008). Several studies have suggested that Th1 cytokines, such as IL-12 and IFN- γ , increase apoptosis (Kodama et al. 2003; De Rose et al. 2004; Tong et al. 2006). These studies also showed that IFN- γ inhibits the proliferation of allergen-stimulated CD4 T cells from atopic, asthmatic patients by inducing surface expression of Fas and Fas Ligand (L), two molecules necessary for activation-induced cell death; thereby triggering an apoptotic program (De Rose et al.

2004). These results are consistent with those obtained by Tong et al. (2006) in a murine model of asthma. Similarly, Kodama et al. (2003) suggested that administration of IL-12 is protective in a Th2-dependent model of allergic airway inflammation, at least partly via enhanced apoptosis of CD4 T cells. However, the apoptotic phenomenon presented in our study was in the setting of acute RAO. Therefore, more studies are needed to examine apoptosis and cytokine profiles in other stages of the disease.

Apoptosis has emerged as a major mechanism in the clearance of activated T cells during the resolution of an inflammatory response (Akbar and Salmon 1997). Inadequate T cell apoptosis in asthma patients appears to interfere with normal T cell elimination, resulting in T cell accumulation, which contributes to chronic inflammation and may be the major underlying cause for tissue damage, remodeling and repair (Müller et al. 2006; Vignola et al. 2000). Spinozzi et al. (1998) reported that pulmonary T cells isolated from BALF of atopic asthma patients showed hypoexpression of Fas and FasL; this result may explain the low frequency of apoptosis in this group of patients. In addition, basal levels of apoptotic activity were significantly lower in BALF from asthmatic subjects compared with peripheral blood lymphocytes from the same subjects. These data indicate that airway inflammation in asthma is associated with a reduced susceptibility to apoptosis, which may lead to enhanced survival of lymphocytes in the bronchial mucosa and prolonged inflammation (Müller et al. 2006). Other molecules are involved in the programmed cell death process, including members of the Bcl-2 gene family, which are known to inhibit apoptosis. Studies have shown that Bcl-2 expression is increased in lymphoid cells obtained from the airways of asthmatic patients and that neutralization of IL-10, an important inducer of Bcl-2, decreases Bcl-2 expression and apoptosis of cells from the respiratory tract of asthmatic patients (Hamzaoui et al. 1999a; Hamzaoui et al. 1999b). Because RAO in horses is a condition similar to human asthma, these apoptotic mechanisms might have an important role in the inflammatory processes described above. Identifying the cellular and molecular mechanisms underlying the reduction of inflammatory cell apoptosis will be crucial for new and effective therapeutic strategies in RAO-affected horses.

In summary, dysregulated apoptosis may play an important role in the pathogenesis of allergic airway inflammation. Our study shows that acute RAO-affected horses have increased apoptosis of airway lymphocytes, which may partially explain the rapid resolution of this pathology once the allergen is removed. Interestingly, this process is in contrast to human asthma, where such cell clearance mechanisms are reduced, leading to chronic inflammation. To that end, further studies will be required to clarify the role of T cell apoptosis in RAO-affected horses.

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