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Increased leishmanicidal activity of alveolar macrophages from mature horses with mild equine asthma

[*Aumento da atividade leishmanicida de macrófagos alveolares de equinos adultos com asma equina leve*]

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ABSTRACT

Alveolar macrophages (AMs) are an essential part of defense mechanisms within the lungs and their phagocytic activity is important for organ homeostasis. The phagocytic ability of AMs obtained from bronchoalveolar lavage from 17 mature mixed-breed pleasure horses (8 healthy and 9 diagnosed with mild equine asthma) was studied through assays with *Leishmania (Viannia) braziliensis* promastigotes, which enabled the calculation of a phagocytic index (PI) and a survival index (SI). Results indicate that phagocytic activity of AMs in asthma affected horses is similar to healthy horses, while leishmanicidal activity is significantly increased in horses with asthma.

Keywords: horses, equine asthma, alveolar macrophage, phagocytosis, *Leishmania (Viannia) braziliensis*

RESUMO

Os macrófagos alveolares (MAs) são uma parte essencial dos mecanismos de defesa dentro dos pulmões e sua atividade fagocítica é importante para a homeostase desse órgão. A capacidade fagocitária dos MAs obtidos do lavado broncoalveolar de 17 equinos adultos, sem raça definida (oito saudáveis e nove com diagnóstico de asma equina leve), foi estudada por meio de ensaios com promastigotas de Leishmania (Viannia) braziliensis. Foi calculado o índice fagocítico e o índice de sobrevivência. Os resultados indicam que a atividade fagocítica de MAs em cavalos com asma é semelhante a cavalos saudáveis, enquanto a atividade leishmanicida está significativamente aumentada em cavalos com essa enfermidade.

Palavras-chave: cavalos, asma equina, macrófago alveolar, fagocitose, Leishmania (Viannia) braziliensis

INTRODUCTION

Alveolar macrophages (AMs) are the front line of cellular defense against respiratory pathogens and inhaled particles in the lungs (Karagianni *et al.*, 2013). Intense exposure to air pollutants is an important risk factor for Inflammatory airways disease (mild equine asthma) in urban policing horses (Lessa *et al.*, 2011). Particle deposition in the alveolar tissue affects the lungs' function (Tetley, 2002), therefore lungs have a particle clearance system to minimize it. This system is highly dependent on phagocytic activity, and both AMs and neutrophils are involved in particle depuration of the lower airway (Oberdörster, 1988; Lehnert, 1992).

The equine AMs' phagocytosis has been studied in healthy horses (Mori *et al.*, 2001; Muehlmann *et al.*, 2012; Karagianni *et al.*, 2013), and in several conditions, including Recurrent Airway Obstruction (RAO) (Olszewski and Laber 1993;

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Tremblay *et al*., 1993; Franchini *et al*., 1998) and Inflammatory airways disease (IAD) (Michelotto *et al.,* 2010), renamed recently to severe and mild equine asthma syndrome, respectively (Couëtil *et al.,* 2016).

Leishmania (Viannia) braziliensis preferentially infects macrophages and the direct relationship between leishmanicidal activity and macrophage activation state is well known in murine macrophages in *Leishmania (Viannia) braziliensis* infection models (Evans *et al.,* 1993, Assreuy *et al.,* 1994 e Mukbel *et al.,* 2007, Giudice *et al.,* 2012, Ghosh *et al.,* 2014). Phagocytosis of *Leishmania (Viannia) braziliensis* promastigotes depends on interactions between macrophage receptors, *Leishmania (Viannia) braziliensis* surface antigens and soluble serum components (Ueno and Wilson, 2012).

Since *Leishmania (Viannia) braziliensis* infects horses in Brazil (Vedovello Filho *et al.* 2008), the protocol of infection of murine peritoneal macrophages (PM) as described by Pinto-da-Silva *et al.* (2002) was used, but murine PM were replaced by equine AMs.

The role of AMs in the pathogenesis of equine asthma syndrome is still poorly defined (Hughes *et al.,* 2011), therefore studying phagocytic and microbicidal capacity of AMs in these horses is yet another step toward understanding the pathogenesis of the condition, as well as to formulate future treatment strategies based on the modulation of macrophage activation status.

In order to investigate alveolar macrophage activation status in horses diagnosed with mild asthma, AMs collected from the airways of healthy and affected horses were cultivated with *Leishmania (Viannia) braziliensis* and their ability to phagocytize and kill the parasites was compared.

MATERIALS AND METHODS

All procedures were approved by the Committee for Animal Care and Use of the Universidade Federal Fluminense, Niterói, Brazil (protocol number 124/2011). All animals were kept in stalls without bedding for 6 to 15 hours per day and the remaining time in paddocks. Horses were fed pelleted feed, grass hay and fresh grass, were vaccinated and dewormed regularly. The daily exercise routine was light to moderate. No horses in either group had undergone treatment for, nor exhibited signs of, airway inflammation in the two months prior to the commencement of the study.

Seventeen mature mixed-breed pleasure horses (6 females and 11 geldings, mean age 15.2±3.4 years) were used in this study. Eight horses belonging to the Brazilian Army were considered healthy (the control group) and nine police horses from Rio de Janeiro, Brazil (PMERJ) were classified as the asthma-affected group, based on clinical examination, tracheobronchoscopy, pulmonary function tests (maximum change in pleural pressure, ΔP_{Plmax}) and bronchoalveolar lavage cytology.

The asthma-affected horses used in this study were mature pleasure horses and may be classified as mildly affected according to criteria previously published (*Lessa et al.,* 2011, Sad *et al.,* 2013, Couëtil *et al.,* 2016). Horses were classified as healthy when they had no abnormal clinical findings, no abnormalities at endoscopy, a $\Delta P_{Plmax} \leq 4$ cm H_2O according to criteria established by Deegen and Klein (1987) and bronchoalveolar lavage fluid (BALF) cytology with neutrophils < 5%, eosinophils < 1% and mast cells < 2% (Mazan, 2010). Horses were included in the asthma-affected group if they demonstrated the following: $\Delta P_{Plmax} \leq 4$ cm H_2O, grade 1 to 2 (Gerber *et al.,* 2004) tracheal secretions, and BALF cytology with neutrophils >5%. Some of the horses in this group (5/9) also demonstrated increased bronchovesicular sounds. All horses had hematology and fibrinogen profiles within normal ranges for the species according to the literature (Jain, 1993).

The AMs were obtained by bronchoalveolar lavage using a catheter (Equine Bronchoalveolar Lavage Catheter®, Bivona, Smiths Medical PM, Inc.) as described by Hoffman and Viel (1997), where bronchoalveolar fluid was obtained by infusing 250mL of 0.9% saline at 37°C into one of the lungs and immediately retrieving it by aspiration with a syringe. The BALF was centrifuged at 450g for 12min at 4°C, and the cell pellet was re-suspended in RPMI 1640 (Gibco BRL) supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Hyclone Lab. Inc. Logan, Utah, USA), 100IU/mL penicillin and 100µg streptomycin/mL. Cell viability

(80%) was determined by Trypan blue exclusion counted in a Neubauer chamber. Bronchoalveolar lavage fluid in RPMI-FCS was seeded at 5×10^6 cells/mL in sterile 13mm coverslips (Glasstécnica®) inside a 24 well cell-culture plate (Costar®) and incubated at 37°C, 5% CO_2 for 1 hour to allow AM adhesion. The coverslips were then washed with sterile PBS at 37°C to remove unattached cells, 300µL RPMI-FCS was added, and cultures were incubated overnight at 37°C at 5% CO_2.

Leishmania (Viannia) braziliensis promastigotes (MHOM/BR94/H3456) were cultivated in Schneider's Insect Medium (Sigma), supplemented with 10% heat-inactivated FCS and 40mg/mL of gentamicin (Sigma), at 26°C for 5–7 days, after which the parasites reached the stationary-phase. The promastigotes were washed with PBS, and centrifuged at 2760g for 10min, three times. The pellet was then re-suspended in RPMI and incubated with AMs at a ratio of 10:1 parasite to AM, and cultures incubated at 37°C, 5% CO2 for 1h to allow parasite uptake and adhesion. For these experiments AMs were assayed after 1h. Cultures were washed with PBS, fixed with methanol, stained with Giemsa and mounted on glass slides with Permount (Fisher Scientific, NJ, USA) after dehydration in acetone/xylene. The number of *Leishmania (Viannia) braziliensis* and the percentage of AMs adhered/phagocytosed to parasite were determined by counting 200 cells in duplicate cultures.

A phagocytic index (PI) for each horse was calculated by multiplying the percentage of AMs which had phagocytosed *Leishmania (Viannia) braziliensis* by the number of parasites per AM as previously described by Pinto-da-Silva *et al.* (2002). All the PIs were then added and divided by the number of horses to obtain the mean PI. In order to determine parasite survival, after 1h of incubation, cultures were washed with PBS to remove free parasites, RPMI-FCS was added and the cells were incubated at 37°C, 5% CO2 for 48h. Results were expressed as a mean percentage of parasite survival (survival index, SI), comparing PIs of each horse after 1h interaction (100%) in relation to 48h, in quadruplicate cultures (Pinto-da-Silva *et al.*, 2002). Data was analyzed by ANOVA at 5% significance level.

RESULTS AND DISCUSSION

To the best of the authors' knowledge the data reported here is the first to compare the phagocytic activity and killing ability of AMs from healthy and asthma-affected mature horses. The percentage of AMs that adhered/phagocytosed *Leishmania (Viannia) braziliensis* (P= 0.17), the number of parasite per AMs (P= 0.50), and PI (P= 0.21) were not significantly different between healthy and asthma-affected horses (Table 1).

Table 1. Phagocytic activity of equine alveolar macrophages

Group	N	Particles adhered/phagocytosed (%) (P= 0.17)	Number of particles/macrophages (P= 0.50)	Phagocytic Index (P= 0.21)
Healthy	8	41.22±13.77[a]	1.86±0.33[b]	80.45±43.10[c]
Asthma-affected	9	32.39±11.67[a]	1.77±0.22[b]	58.73±25.53[c]

Results are expressed as mean±standard deviation (SD). In order to obtain the mean phagocytic index (PI), the PIs of all horses were added and divided by the number of horses. Same letters in the same column indicate lack of statistically significant differences (p-values are indicated in the table).

Phagocytosis depends on engagement of particles through receptor-mediated binding which precedes internalization and induction of cellular antimicrobial responses (Rieger *et al.*, 2010) and in the case of *Leishmania (Viannia) braziliensis* promastigotes it depends on interactions between macrophage receptors (CR1, CR3, MR and FnR), *Leishmania (Viannia) braziliensis* surface antigens (GP63, LPG and an

unknown ligand) and soluble serum components, such as: C3, C3b, iC3b, Factor I and Fibronectin (Ueno and Wilson, 2012). It can be hypothesized that the lack of differences in macrophage response between healthy and asthma-affected horses seen in the present work indicates that the asthmatic state does not promote a change in AMs surface receptors.

The leishmanicidal activity of murine macrophages is directly related to their activation state and production of NO and intermediate reactive oxygen radicals such as peroxide (Evans *et al.*, 1993; Assreuy *et al.*, 1994; Giudice *et al.*, 2012, Mukbel *et al.*, 2007, Ghosh *et al.*, 2014). Nitric oxide (Michelotto *et al.*, 2010) and peroxide (Mori *et al.*, 2001) were previously detected in equine AMs and were related to increased activation state.

In this work it was not possible to detected NO and peroxide (data not shown) to confirm activation state. The observed decrease in parasite SI in the AMs after 48h was higher (P= 0.0041) in asthma-affected horses than in healthy ones (Table 2). It was likely due to high microbicidal activity, which represents a higher activation state of the cells in this group, as previously seen for murine macrophages (Evans *et al.*, 1993; Assreuy *et al.*, 1994; Giudice *et al.*, 2012, Mukbel *et al.* 2007, Ghosh *et al.*, 2014). Results from the present work indicate that phagocytic capacity for *Leishmania (Viannia) braziliensis* is unaltered in horses with mild asthma while leishmanicidal activity is significantly increased.

Table 2. *Leishmania (Viannia) braziliensis* survival index (SI) in equine alveolar macrophages

Group	N	*Leishmania* SI
Healthy	8	43.1 ± 19.70^{b}
Asthma-affected	9	18.15 ± 9.77^{a}

Results are expressed as mean±SD of parasite survival comparing phagocytic indices of each horse after 1h interaction (100%) in relation to 48h, in quadruplicate cultures. Different letters in the same column mean the differences are statistically significant (ANOVA, P= 0.004).

The activation status of AMs in healthy horses was previously reported (Mori *et al.*, 2001). Since exposure to dust has already been described as a cause of stimulation of alveolar macrophages (Franchini *et al.*, 1998), it is expected that AMs might be already activated, even in healthy animals. This reflects the microenvironment within which these cells reside, and their inherent biological properties within such an environment. Unlike peritoneal macrophages (PMs), AMs from healthy horses have demonstrated efficient phagocytic activity for marked *E. coli* particles, and they are more activated than the PMs when stimulated with pro-inflammatory binders (Karagianni *et al.*, 2013). A specific particle challenge was not conducted in this study; therefore, it is not possible to establish if airborne particle exposure resulted in direct macrophage function shift or in inflammation which in turn shifted macrophage function secondarily. In order to investigate these aspects, AM function after particle challenges and in natural viral or bacterial infection could be studied.

The difference in SI between healthy AMs and those obtained from horses with cytology compatible with airway inflammation should be further investigated using different methods than those described in this research before definite conclusions can be drawn.

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