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Participation of purines in the modulation of inflammatory response in rats experimentally infected by *Cryptococcus neoformans*

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ABSTRACT

The present study was carried out to assess the participation of purines in the activation and modulation of inflammatory response of rats experimentally infected by *Cryptococcus neoformans*. Twenty four Wistar rats were divided into two groups of 12 animals each: Group A - uninfected control group and Group B - infected by *C. neoformans*. Blood was collected 20 and 50 days post-infection (PI) from six animals of each group in order to verify purine levels (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XAN) and uric acid (URIC)). ATP levels were significantly increased ($P < 0.05$) in serum from infected animals on days 20 and 50 PI, as well as adenosine levels after 20 days PI on rats. On day 50 PI, levels of adenosine and uric acid were also reduced, but the levels of inosine and xanthine increased in animals infected by the fungus ($P < 0.05$). Therefore, it was possible to conclude that the purine levels in serum were altered and that these changes may be able to influence the pathogenesis of the disease caused by *C. neoformans* due the participation of purines (ATP and adenosine main) in the activation and modulation of inflammatory response.

1. Introduction

Cryptococcus neoformans, the predominant etiological agent of cryptococcosis, is an encapsulated fungal pathogen that causes disease that ranges from an asymptomatic infection to a mild bronchopneumonia and even to a life-threatening infection of the central nervous system (CNS) [1]. The most common and serious type of cryptococcal disease is the pulmonary cryptococcosis that if uncontrolled may progress to cryptococcal meningitis (CM) or meningoencephalitis [2,3]. Pulmonary cryptococcal infection will occur only when fungal cells are deposited deep into the lungs after inhalation. In the lungs alveolar macrophages (AMs) will be the first cells to encounter the fungus and will respond by internalizing them through phagocytosis [4]. When cryptococcal infection is not controlled at this stage, the microorganism may spread throughout the body, with particular preference for the CNS leading to meningitis and/or meningoencephalitis, a life-threatening disease with high mortality rates [1,5].

Resistance or susceptibility to *Cryptococcus* disease in humans and animals depend on the outcome of many host and pathogen derived factors. In this sense, purinergic mechanisms have been shown to be involved in various pathological conditions where

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nucleotides ATP, ADP, AMP and the nucleosides adenosine and inosine are secreted by hematological and endothelial cells and used as mediators able to modulate the inflammation process, vascular thrombosis, muscle contraction, neurotransmission and pain [6–9]. Additionally, purinergic mechanisms also play an important role in brain trauma and ischaemia, neurodegenerative diseases involving neuroimmune and neuroinflammatory reactions, as well as in neuropsychiatric diseases, including depression and schizophrenia [8]. The deamination of adenosine to inosine favors the maintenance and survival of invading microorganisms, since adenosine promotes chemotaxis, activation and degranulation of mast cells [10,11]. Hypoxanthine, xanthine, and uric acid are bioproducts of purine catabolism [12], being the uric acid a potent antioxidant. Therefore, the aim of this study was to access the purines levels of the purinergic cascade in serum samples from rats experimentally infected by *C. neoformans* and to evaluate the participation of purines in the modulation of inflammation.

2. Materials and methods

2.1. Animals

Twenty-four adult male rats with 90 days of age and 274 (±21) grams of weight were used. They were kept in cages with six animals each on an experimental facility under controlled temperature and humidity (25 °C; 70%), fed with commercial feed, with water *ad libitum,* and submitted to a period of 12 days for adaptation.

This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (UFSM), under protocol number 6598280615.

2.2. Strain

2.2.1. C. neoformans var. grubii

C. neoformans used for inoculum preparation was obtained from a clinical case of a feline with cryptococcosis [13]. This strain had previously been identified as *C. neoformans* var. *grubii.* The genomic sequence obtained was deposited GenBank under the accession number HQ148880.

2.2.2. Inoculum preparation

C. neoformans was grown in Sabouraud dextrose agar (SDA) medium for 24 h at 30 °C to obtain a viable and pure strain. The fungal inoculum was prepared using young colonies diluted in phosphate-buffered saline (PBS).

2.3. Experimental design

In this study, Wistar rats were divided into two groups: 12 animals composed the control group A (group: uninfected), and 12 animals composed group B (group: infected) infected by *C. neoformans*. After isoflurane anesthesia, the inoculation of 0.3 mL containing 1.7×10^7 cells of *C. neoformans* was performed intratrachealy as described previously [14].

2.4. Course of infection

Course of infection was assessed by the evaluation of colony-forming units (CFU) on days 20 and 50 PI (sampling times). The lungs of rats were collected and weighed after euthanasia. A fragment of each organ was mechanical grinded and homogenized in PBS (1:10) containing 40 U of penicillin/mL. All samples were submitted to serial dilutions and 100 μL aliquots were added to plates containing SDA and incubated at 30 °C for 24–72 h. Data were reported as CFU/g of organ analyzed. The results of CFU were shown as mean and standard deviation.

2.5. Collecting samples

Samples from the groups A and B were collected on days 20 and 50 PI as follows: on day 20 (six animals of group A and six animals of group B); and on day 50 (six animals of group A and six animals of group B). Blood samples were collected by cardiac puncture (4 mL) from anesthetized animals. Blood samples were allocated in tubes without anticoagulant in order to obtain serum to measure the levels of purines. To obtain the serum, all blood samples were centrifuged (5000 g for 5 min at 37 °C). The serum was stored at −20 °C until analysis.

2.6. Serum preparation

The denaturation of serum sample proteins was performed using 0.6 mol L^{-1} perchloric acid. All samples were then centrifuged (14000 g for 10 min at 4 °C) and the supernatants were neutralized with 4.0 N KOH and clarified with a second centrifugation (14000 g for 15 min at 4 °C). The serum of rat from each group (A and B) was used to measure the concentration of purines (ATP, ADP, AMP, ADO, INO, HYPO, XAN and URIC) [15].

2.7. Analysis of purine levels in serum

Aliquots of 20 μL of serum samples were applied to a reversed-phase performance liquid chromatography (HPLC) system (Shimadzu, Japan) using a C_{18} column (Ultra C18, 25 cm × 4.6 mm x 5 μm, Restek - USA). The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30 min period (flow rate at 1.4 mL/min) according to a method previously described [16]. The amounts of purines were measured by absorption measured at 260 nm. The retention time of standards was used as parameter for identification and quantification. Purines concentrations were expressed as nmol of different compounds per mL of serum.

2.8. Histopathology

Lungs tissues from three animals of each group (three of each period evaluated, days 20 and 50 PI) were collected, and then stored in 10% formalin buffered solution. Sagittal sections of every 3 mm were obtained and stained with hematoxylin and eosin.

2.9. Statistical analysis

The data were submitted to one-way analysis of variance followed by the Students t-test ($P < 0.05$). The values were presented as mean ± standard error. All samples were processed in triplicate.

3. Results

3.1. Course of infection and clinical signs

Rats showed mild apathy and piloerection, and one rat died before the end of the experiment with macro-lesions in the lungs, and histological examinations revealed the presence of fungus associated with pneumonia. Similar findings were also found on other experimental animals described below.

C. neoformans was isolated from the lungs of infected animals on days 20 and 50 PI, resulting in the growth of white mucoid yeast