Pathology – Research and Practice 211 (2015) 851–857

Contents lists available at ScienceDirect

Pathology – Research and Practice

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

Original Article

Cholinesterase of rats experimentally infected by *Cryptococcus neoformans*: Relationship between inflammatory response and pathological findings

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ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 18 May 2015
Accepted 3 August 2015

Keywords:
Cryptococcosis
Acetylcholine
Acetylcholinesterase
Butyrylcholinesterase
Inflammatory process
Oxidative stress

ABSTRACT

The aim of this study was to assess the role of the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as biomarkers of inflammation and tissue injury on rats experimentally infected by *Cryptococcus neoformans*. For this purpose, 20 male rats were divided into two groups: 10 animals representing the uninfected control group (Group A) and 10 *C. neoformans* var. *grubii* infected animals (Group B). Blood and brain samples were collected on days 10 (A_{10} and B_{10}), and 30 (A_{30} and B_{30}) post-infection (PI) for hematological analyses; AChE (in lymphocytes and brain) and seric BChE activity; interleukins (IL-1, IL-6, and IL-10); nitrite/nitrate (NO_x) levels; and markers of protein oxidation (AOPP) and lipid peroxidation (TBARS). As a result, when animals of Group A were compared to animals of Group B, it was observed leukocytosis ($P<0.05$) on day 10 PI; AChE activity increase ($P<0.05$) in lymphocytes (day 30 PI) and in brain (days 10 and 30 PI); BChE activity decrease ($P<0.05$) on day 10 PI; IL-1 and IL-6 increase ($P<0.01$) in both periods, while IL-10 had reduced levels ($P<0.01$) in the same periods; NO_x levels increased ($P<0.05$) significantly on days 10 and 30 PI, while AOPP and TBARS levels increased significantly on day 30 PI; as well as pneumonia on infected rats. Therefore, based on the results obtained, it was possible to conclude that AChE and BChE behavior lead to a proinflammatory reaction evidenced by the enhancement of IL-1, IL-6, and NO_x throughout the experiment associated with reduction on IL-10 levels, and cellular damage.

1. Introduction

Cryptococcus neoformans is an encapsulated yeast and the causative agent of opportunistic mycoses called cryptococcosis, an infection predominantly occurring in immunocompromised hosts [1]. The infection occurs mainly in the respiratory tract, caused by inhalation of contaminated dust and debris, spreading from the lungs to other organs, especially the central nervous system (CNS) [2]. The immune defense against pathogens usually causes an inflammatory response associated with an anti-inflammatory response [3]. In this sense, studies indicate that passive antibody-mediated protection against *C. neoformans* requires both Th1 and Th2 associated cytokines, and reveal the complexity of the mechanisms through which antibodies modulate infection with this type of microorganism [4].

Regarding the mechanisms of immune response associated with the predilection of *C. neoformans* for the CNS, it is important to emphasize that the autonomic nervous system plays an

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http://dx.doi.org/10.1016/j.prp.2015.08.005
0344-0338/

important role in controlling inflammation partially by the release of pro-inflammatory cytokines, through the secretion of adrenaline and noradrenaline, and by connecting these to α and β androgenic receptors on the immune cells [5]. In contrast, the vagal efferent pathway plays a very important role, modulating the inflammation and, for that, being called cholinergic anti-inflammatory pathway [3,5]. The main vagal neurotransmitter is acetylcholine (ACh), which is catalyzed by the cholinergic enzymatic system, composed of the cholinesterases. This group of enzymes is composed of acetylcholinesterase (AChE: E.C. 3.1.1.7), a membrane-bound enzyme mainly found in the brain, muscles, erythrocytes, lymphocytes and cholinergic neurons, that preferentially hydrolyzes esters with acetyl group [6,7] and butyrylcholinesterase (BChE: E.C. 3.1.1.8). BChE is a serine hydrolase that catalyzes the hydrolysis of esters of choline such as butyrylcholine and succinylcholine, found at different concentrations in the intestine, liver, kidney, heart, lung, brain, and serum [8,9]. ACh has an important role in attenuating the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-18 (IL-18), and on endotoxin-activated macrophages without affecting the production of interleukin-10 (IL-10), an anti-inflammatory cytokine [5]. This inhibition is possible by ACh binding to α7 subunit-containing nicotinic acetylcholine receptor (α7nAChE) present on macrophages, being the dose-dependent inhibition [10].

In association with the evaluation of the participation of cholinesterases in the immune response against *C. neoformans*, some strategies to measure tissue and/or system damage, such as biomarkers of oxidative stress would be useful tools since they have the potential to help in the establishment of pathogenic stages, increasing the risk for disease [11–13]. Among these biomarkers, the assessment of thiobarbituric acid reactive substances (TBARS) is one of the most commonly applied methods for the measurement of lipid peroxidation [14], and the measurement of advanced oxidation protein products (AOPP) is another useful option. AOPP also helps to identify oxidative damage; however, specifically for proteins [15]. In this sense, another possibility is the measurement of nitric oxide (NO) levels, which allows the evaluation of both the immune responses [16] and oxidative stress status [17], acting as an extra tool for diagnosis and disease treatment [18,19].

Therefore, considering all the information mentioned above, the aim of this study is to evaluate the role of the cholinesterases as markers of inflammation and tissue injury in rats experimentally infected by *C. neoformans*.

2. Materials and methods

2.1. Animals

Twenty male Wistar rats (*Rattus norvegicus*) with 90 days of age and 298 (±29) g of average weight were kept in cages housed in a room with controlled temperature and humidity (25 °C; 70%). They were fed with commercial ration and received water *ad libitum*. All animals had a period of 7 days for adaptation and were clinically healthy in the beginning of the experiment (day 0). The procedure was approved by the Committee on Ethics in Animal Experimentation of Universidade Federal de Santa Maria (protocol number 073/2012).

2.2. Cryptococcus neoformans *var.* grubii *strain and inoculum*

2.2.1. Cryptococcus neoformans *var.* grubii

The strain of *C. neoformans* used for animal inoculation in this study was obtained from a clinical case of feline cryptococcosis [20]. This strain was previously identified as *C. neoformans* var. *grubii* based on its micromorphology and molecular characteristics, as well as by its genomic sequence data deposited in GenBank under the accession number HQ148880.

2.2.2. Inoculum preparation

C. neoformans was isolated on Sabouraud dextrose agar (SDA) and incubated at 32 °C in order to obtain a pure and viable strain. Each animal was inoculated with 0.3 ml of a suspension containing 1.7×10^7 cells of *C. neoformans* [21]. The inoculum was prepared from young fungal colonies diluted in phosphate-buffered saline (PBS).

2.3. Experimental design and infection

Rats were divided into two groups: 10 animals for the control group (Group A: uninfected) and 10 *C. neoformans* var. *grubii* infected animals (Group B). The inoculation was performed as previously described [21]. For inoculation, all animals were anesthetized with isoflurane. All animals underwent daily clinical evaluation throughout the experiment.

2.4. Progression of infection

Progression of infection was assessed by the evaluation of colony-forming units (CFU) at days 10 and 30 PI (sampling times). Briefly, brain and lungs of rats were collected and weighed after euthanasia. The organs were homogenized for 5–10 s in PBS containing 40 U of penicillin per 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.

2.5. Sampling

Blood samples were collected on days 10 and 30 PI from five anesthetized (isoflurane) animals of each group by cardiac puncture. Blood samples were stored in tubes containing anticoagulant (for separation of lymphocytes and hemogram) and in tubes without anticoagulant (to obtain sera to determine BChE activity and interleukins levels).

Brain samples from five rats of each group were also collected on days 10 and 30 PI, and they were divided into two hemispheres. The right hemisphere was weighed and homogenized in buffer Tris-HCl 10 mmol, pH 7.2 to verify the activity of AChE, along with NO_x, AOPP, and TBARS levels. All procedures described above were performed under refrigeration temperature (4 °C). Homogenates were stored and frozen at −20 °C until analyses. The left hemisphere was stored in 10% formalin for histological analyses.

2.6. Hematology

Hematocrit, total erythrocytes, hemoglobin concentration, and total leukocytes were evaluated using an automatic electronic counter. Smears were set and stained by the panoptic method to perform differential leukocyte counts. The hematocrit was obtained by centrifugation using a microcentrifuge (Sigma) at 18,600 × *g* for 5 min [22].

2.7. Cholinesterases activities

2.7.1. AChE in lymphocytes

Lymphocytes were obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus, according to the technique described by Böyum [23]. Lymphocyte viability and integrity were confirmed by determining the percentage of cells