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Cytokines in rats experimentally infected with *Trypanosoma evansi*

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ABSTRACT

The aim of this study was to measure the levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) in the serum of rats experimentally infected with *Trypanosoma evansi* and to correlate these levels with hematological parameters. Initially, 48 rats (group T) were intraperitoneally inoculated with cryopreserved blood containing 1×10^6 trypomastigotes per animal. Twenty-eight animals (group C) were used as negative controls and received 0.2 mL of saline by the same route. The experimental groups were formed according to the time after infection and the degree of parasitemia as follows: four control subgroups (C3, C5, C10 and C20) with seven non-inoculated animals each and four test subgroups (T3, T5, T10 and T20) with 10 animals each inoculated with *T. evansi*. The blood samples were collected by cardiac puncture at days 3 (C3, T3), 5 (C5, T5), 10 (C10, T10) and 20 (C20, T20) post-infection (PI) to perform the complete blood count and the determination of IFN-γ, TNF-α, IL-1 and IL-6 levels using an ELISA quantitative sandwich. Infected rats showed normocytic normochromic anemia during the experimental period. *T. evansi* infection in rats caused a serum increase ($P < 0.01$) of IFN-γ, TNF-α, IL-1 and IL-6 levels at days 3, 5, 10 and 20 PI compared to the controls. The multiple linear regressions showed a reduction of 24% in the hematocrit as a consequence of the increased IFN-γ, TNF-α and IL-1. Therefore, we conclude that the infection caused by *T. evansi* causes an increase in the pro-inflammatory cytokines. These results suggest a synergism among IL-1, TNF-α and IFN-γ contributing to the development of anemia. This increase is associated with the regulation of immune responses against the parasite.

1. Introduction

Trypanosoma evansi is a pathogenic hemoflagellate protozoa belonging to the Salivaria section, which presents global distribution and affects several animal species. Disease progression and the clinical, hematological and pathological aspects in the host can vary according to the strain's virulence, host susceptibility and epizootic conditions. Transmission occurs mainly by hematophagous insects (*Tabanus* sp., *Chrysops* sp. and *Hematopota* sp.) (Hoare, 1972).

After reaching the lymphatic system, *T. evansi* leads to a B and T cell response depending on the type of parasite cell surface molecule (variant surface glycoprotein – VSG) (Taylor and Authié, 2004). Most of the immunoglobulins produced in response to the infection belong to IgM and IgG classes. These antibodies bind to specific epitopes on the parasite's surface causing its death (Baral et al., 2007). Costa et al. (2010) reported an increase in the total serum protein levels in cats infected by *T. evansi* corresponding to alpha-2-globulin, beta-globulin and serum gamma-globulin fractions. This is reported to be related with the host response in the inflammatory process. The immune system is involved in infection control and activates different cellular mechanisms, in which cytokines play an important role.

Cytokines are low molecular weight proteins which act as intercellular mediators involved in many biological processes such as inflammation, fibrosis, angiogenesis, cell growth/proliferation and immune response (Tizard, 2002; Feldmann, 2008). In trypanosomosis the lymphocytes produce interferon-gamma (IFN-γ) in response to parasite antigens. IFN-γ activates the macrophages increasing their ability to destroy phagocyted organisms. The activated macrophages induce the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α playing an important role in the replication process of the parasite as well as in the host

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immune response (Paulnock and Coller, 2001; Gao and Pereira, 2002; Magez et al., 2007). Cytokines, in addition to their well documented roles, are referred to have suppressor activity on erythropoiesis (Cançando and Chiattone, 2002) and probably are central players in anemia associated with inflammation (Noyes et al., 2001).

Unlike other trypanosomosis, information about the immunological mechanisms playing a role in *T. evansi* infection is limited. There is no data regarding the serum levels of inflammatory cytokines such as IFN-γ, TNF-α, IL-1 and IL-6 in infected animals. The aim of this work was to provide information about the serum levels of these cytokines in *T. evansi* infection and to establish a correlation with hematological parameters.

2. Material and methods

2.1. Experimental animals

Seventy-six Wistar rats (*Rattus norvegicus*; 230–270 g) were housed in a room with controlled temperature (23 °C) and relative humidity (70%). The rats were fed with commercial rat pellets and received water *ad libitum*.

This study was approved by the Ethics and Animal Welfare Committee of the Rural Science Center of the Federal University of Santa Maria (CCR/UFSM), No. 23081.014568/2009-05 in accordance with existing legislation and the Ethical Principles published by the Brazilian College of Animal Experiments (COBEA).

2.2. Groups and trypanosome infection

Rats were divided into two groups as follows: group T was constituted by 48 Wistar rats inoculated with *T. evansi* strain and group C formed by 28 animals used as negative controls. For this experiment, a strain of *T. evansi* obtained from a naturally infected dog was used (Colpo et al., 2005), maintained in liquid nitrogen at the laboratory according to the methodology described by Silva et al. (2003). At day zero the parasites were thawed and the number of trypanosomes per mL was determined using a hemocytometer under microscope (Wolkmer et al., 2007). The animals from group T were inoculated intraperitoneally with cryopreserved blood (0.2 mL) containing 1×10^6 trypomastigotes per animal. The control animals received 0.2 mL of sterile saline (0.9% NaCl) in the same way.

The two groups were divided into four subgroups each, organized according to the time of infection and the degree of parasitemia. Four subgroups defined as controls (C3, C5, C10 and C20), each group composed of seven non-inoculated animals; and the test groups (T3, T5, T10 and T20), inoculated with *T. evansi*, were formed by 10 animals each.

2.3. Estimation of parasitemia

The presence and degree of parasitemia were estimated for each animal daily by blood smear examination. A drop of blood was collected from the tail vein and placed on a slide, and a thin blood smear was manually prepared. The blood films were stained with the Romanovsky stain and then examined under a microscope, the parasites were counted in 10 fields at 1000× magnification.

2.4. Blood sampling

The blood samples were collected at days 3 (C3, T3), 5 (C5, T5), 10 (C10, T10) and 20 (C20, T20) post-infection (PI). For blood collection (5 mL) by heart puncture, the rats were anesthetized with isofluorane in a gas chamber. After the blood sampling, the rats were sacrificed by exsanguinations under anesthesia. For complete blood count (CBC), 1 mL of blood from each rat was placed in tubes containing 10% ethylene diamine tetraacetic acid (EDTA). The remaining 4 mL was transferred into tubes without anticoagulants and centrifuged to obtain the serum, addressed for the quantification of IFN-γ, TNF-α, IL-1 and IL-6.

2.5. Hematological evaluation

Complete blood count (CBC) and hemoglobin (Hb) determination were performed using an automated cell counter (Vet Auto Hematology Analyzer®, model BC 2800). The packed cell volume (PCV) was obtained by centrifugation using a microcentrifuge (Sigma) at 14.000 rpm for 5 min. For morphological evaluation of the blood and differential count of the white blood cells (WBCs), the blood smears were first stained using a Diff-Quick commercial kit and subsequently visualized under the microscope. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Feldman et al. (2000).

2.6. Cytokines

Pro-inflammatory cytokines quantification was assessed by ELISA using commercial kits for rat IFN-γ, TNF-α, IL-1 and IL-6 (eBIOSCIENCE®, San Diego, USA), according to manufacturer's instructions. Briefly, 96 well microplates were sensitized with the primary antibody at room temperature (RT) for 30 min, then the sample was added and incubated (37 °C temperature, for 30 min). After washing, the secondary antibody conjugated with peroxidase was added and incubated. The presence and concentration of the cytokines were determined by the intensity of the color measured by spectrometry in a micro ELISA reader.

2.7. Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) and the means were compared using Tukey's test. In order to verify the effects of the cytokine levels on the PCV, multiple linear regression (MLR) using the hierarchical method for TNF-α and IL-1 was chosen. The MLR stepwise method was used for IL-6 and IFN- γ. *P* values lower than 5% were considered to be statistically different.

3. Results

3.1. Parasitemia and clinical course of infection

T. evansi could be detected in the blood of all infected rats from 24 to 72 h after inoculation. Parasitemia levels increased progressively in most animals until day 5 PI, when the first peak of parasitemia was observed (mean of 62 trypanosomes/HPF). In this first peak of parasitemia, eight infected rats maintained a progressive increase in the number of parasites in the blood and died between days 5–7 PI with high parasitemia (more than 100 trypanosomes/HPF). After day 6 PI, the remaining rats from subgroups T10 and T20 showed a reduction in parasitemia, which oscillated from 0 to 5 parasites/HPF until day 20 PI. During this experiment, the second peak of parasitemia was not observed, which is usually fatal in rats.

At the time of sample collection, parasitemia of the rats was an average of 28 ± 5.4 trypanosomes/HPF at day 3 PI (T3), 54 ± 12.8 trypanosomes/HPF at day 5 PI (T5), 2.2 ± 1.4 trypanosomes/HPF at day 10 PI (T10) and 1.1 ± 0.9 trypanosomes/HPF at day 20 PI (T20). The eight animals which died showed ataxia, disorientation