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Role of acute phase proteins in the immune response of rabbits infected with *Trypanosoma evansi*

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ABSTRACT

The aim of this study was to characterize the response of acute phase proteins (APP) in rabbits experimentally infected with *Trypanosoma evansi* (*T. evansi*), and to relate the findings with serum immunoglobulins levels, in order to verify the relation between APP and the immune response of rabbits. A total of 12 animals were used in this experiment and divided into 2 groups, control and infected, of six rabbits each. The experimental period was 118 days, and blood was collected on days 0, 5, 20, 35, 65, 95 and 118 post-infection (PI). The infection with *T. evansi* stimulated APP and immunoglobulins production, once the infected animals showed an increase in C-reactive protein, haptoglobin, alpha 2-macroglobulin and IgM levels. The elevation in IgM levels observed in this study, when related to the increase in C-reactive protein and haptoglobin levels, suggests the involvement of these proteins in host defense against flagellated protozoa, with possible participation in the control of the parasitemia in rabbits infected with *T. evansi*.

1. Introduction

Trypanosomosis is a worldwide disease and in Brazil is known as "mal das cadeiras" due to the clinical signs observed in infected animals. Horses are the most affected species in Pantanal Matogrossense region (Brazil) and the most common presentation that characterizes the disease is incoordination of the hind limbs (Herrera et al., 2004; Silva et al., 1995, 2002). The disease is caused by a protozoan, *Trypanosoma evansi* (*T. evansi*), and in rabbits, the clinical signs are similar to those observed in other species, such as weight loss and anemia (Da Silva et al., 2008b; Uche et al., 1992), hyperproteinemia due to hypergammaglobulinemia, and increased alanine aminotransferase and alkaline phosphatase activities (Da Silva et al., 2007). Therefore, rabbits are a good experimental model to study this disease.

Previous studies demonstrated that rabbits may be resistant to the parasite infection (Da Silva et al., 2008a; Uche et al., 1992), which is attributed mainly to immunoglobulins IgG, IgM and IgA (Uche et al., 1993). However, Uche and Jones (1993), related the importance of innate immunity modulating the response mediated by antibodies. In this study, the complement system was depleted in one group of infected rabbits with *T. evansi*. The challenged group, with the complement system active, revealed a more effective response by antibodies than the animals who presented depletion of the system (Uche and Jones, 1993).

Beyond the complement system, there are other proteins, named acute phase proteins (APP) that also assist in the body's defense. APP are released predominant by the liver, as a result of the action of cytokines (IL-1, TNF-α and IL-6) on hepatocytes (Paltrinieri, 2007; Tizard, 2002). These proteins are important components of the immune system, and are involved in the restoration of homeostasis and reduction of microbial growth, before the animals develop acquired immunity (Murata et al., 2004).

It is still not well defined how these proteins are related to the innate and specific response against *T. evansi*, as well as which proteins are involved in this response. Thereby, the aim of this study was to determine the response of APP in rabbits experimentally

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infected with *T. evansi*, evaluating total proteins and its fractions, measuring four acute phase proteins, in addition to immunoglobulins IgM and IgG, correlating the results of the APP levels with immunoglobulins, to verify the importance of APP on the immune response of rabbits.

2. Materials and methods

2.1. Animals

Twelve female adult New Zealand rabbits, weighing 3.6–4.5 kg were used in this study. The animals were maintained in individual cages, at a constant temperature and humidity (23 °C and 70%, respectively), with free access to food and water. All these rabbits were treated with a combination of anthelmintics pyrantel pamoate, praziquantel and febantel, and were submitted to an adaptation period of 30 days. During this period, hematological (CBC) and biochemical (hepatic and renal function) exams were performed with an interval of 15 days, in order to verify the health status of the animals. In both time points, the rabbits presented hematological and biochemical values within the normal range (Campbell, 2007).

The procedure was approved by the Animal Welfare Committee of University Federal de Santa Maria, number 23081.020268/2008-76, in accordance to Brazilian laws and ethical principles published by the Colégio Brasileiro de Experimentação Animal.

2.2. Experimental groups and T. evansi infection

Healthy rabbits were divided into two groups, control and infected, with six animals each. They were inoculated intraperitoneally with 0.5 mL of rat blood containing 1×10^8 trypanosomes per animal, and the control group received saline solution alone by the same route. The *T. evansi* strain used in this study was isolated from a naturally infected dog (Colpo et al., 2005), and the quantification of the injected dose was undertaken using a Neubauer chamber (Wolkmer et al., 2007).

2.3. Estimation of parasitemia

After inoculation, the parasitemia was monitored daily by microscopic examination of blood smears. The peripheral blood was obtained from the ear vein, stained with Panotic® kit and examined under microscope at 1000× magnification.

2.4. Blood sampling

The experimental period was 118 days. The blood samples were collected at days 0, 5, 20, 35, 65, 95 and 118 post-infection (PI), by cardiac puncture using a 5 mL syringe and a 25 × 7 needle, and stored without anticoagulant. For this procedure the rabbits were anesthetized with ketamine (30 mg/kg) and xylazine (4 mg/kg) via intramuscular injection. After collection, the blood was rested for 30 min to allow clot retraction, and then centrifuged at 3.400 rpm for 10 min to obtain the serum, which was separated and stored in microtubes at −20 °C until assayed. After 118 days PI, animals were euthanized using the same protocol described earlier, but the doses were tripled, as recommended by Taborda et al. (2004).

2.5. Determination of total proteins and protein fractionation by electrophoresis

Total proteins were determined using commercial reagents (Labtest®) and analyzed in an automatic biochemical analyzer (Bioplus®200). The protein fractionation was determined using cellulose acetate strip electrophoresis in a horizontal cube (Labex®), with Tris–glycine buffer (pH 8.6), adapted by technical of Naoum (1999). Samples were applied to the strips and run using a constant voltage of 180 volts for 25 min. Strips were stained with Ponceau for 15 min. The excess stain was removed by washing the strips in 5% acetic acid until background was completely clear. Then strips were fixed in methanol for 30 s and washed for 1 min with a destain solution. Strips were dried at 60 °C for 15 min and read by the Denscan system. The fractions analyzed were the albumin, alpha-1, alpha-2, beta-1, beta-2 and gamma globulin.

2.6. Determination of immunoglobulins IgM and IgG

Immunoglobulins IgM and IgG were determined using ELISA commercial kits (Quantitation Set®) according to the manufacturer's instructions. Sera were processed at a dilution of 1:3600 and were compared with the results obtained in the calibrator curve ($R^2 > 0.93$). Each sample was performed in triplicate.

2.7. Determination of acute phase proteins (APP)

The choice of APP described in this study was based on research conducted by Kostro et al. (2003), who describes C-reactive proteins, haptoglobin, alpha-2-macroglobulin and transferrin as the major acute phase proteins in rabbits. Also APP were determined using ELISA commercial kits (Kamiya Biomedical Company®), according to the manufacturer's instructions.

2.8. Statistical analysis

As data presented normal distribution, proven by the Kolmogorov Smirnov test, they were submitted to *t* Student test for comparison between groups. Pearson correlation test was performed to assess the correlation between APP and immunoglobulins. Data were considered significantly different at $P < 0.05$. All statistical analysis were performed with SPSS® program version 17.0.

3. Results

3.1. Parasitemia

Examination of the peripheral blood smears showed a prepatency period between 24 and 72 h in the infected rabbits. Irregular waves of parasitemia, ranging from zero to one trypomastigote per microscopic field, were observed during 35 days PI. Parasites were no longer observed in blood smears from the 37th day onwards [published data in the study of Costa et al. (2012), that is related to this study].

3.2. Total protein and protein electrophoresis

In infected group, total protein levels increased from day 20 PI (Fig. 1A) until day 118 PI, the last day of experiment. Protein electrophoresis revealed hypoalbuminemia in the infected group only at day 5 PI (Fig. 1B). Alpha-1 globulin fraction increased from day 65 PI (Fig. 1C) and high values of alpha-2 globulin fraction ($P < 0.05$) were observed at days 35, 65, 95, and 118 PI (Fig. 1D), accompanied of an increase in beta-1 globulin fraction ($P < 0.01$), that presented higher levels until day 95 PI, when compared to control group (Fig. 1E). Both fractions, beta-2 globulin and gamma globulin, were elevated from the fifth day of infection (Fig. 1F). Beta-2 globulin levels remained higher in the infected group than in control group until day 65 PI, and gamma globulin fraction until day 118 PI (Fig. 1G).