Veterinary Parasitology 172 (2010) 16–22

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Pathogenic action of *Plasmodium gallinaceum* in chickens: Brain histology and nitric oxide production by blood monocyte-derived macrophages

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ARTICLE INFO

Article history:
Received 18 December 2009
Received in revised form 13 April 2010
Accepted 27 April 2010

Keywords:
Plasmodium gallinaceum
Chickens
Cerebral malaria
Nitric oxide
Macrophage activation

ABSTRACT

Plasmodium infection causes major losses to animal and human populations. The characterization of experimental malaria models is needed for a better understanding of disease mechanisms and the development of new treatment protocols. Chickens infected with *Plasmodium gallinaceum* constitute an adequate malaria model due to the phylogenetic proximity of this parasite to human *Plasmodium* as well as similarities in disease manifestation, such as cerebral malaria. The aim of the present study was to further characterize the experimental chicken model with an emphasis on clinical manifestations, cerebral histology and nitric oxide (NO) produced by macrophages. The results revealed that mortality was correlated to higher parasitemia. Parasitemia was positively correlated to temperature and negatively correlated to haematocrit value. Brain histology of infected birds revealed inflammatory infiltrates and blocked microvasculature. Macrophages derived from blood monocytes produced NO after activation, with a higher production positively correlated to parasitemia. These results characterize histological aspects of chicken brain malaria and demonstrate the activation of the innate immune system caused by the infection in chickens.

1. Introduction

A number of studies on the complex physiopathology of malaria have been published, but the cellular and molecular mechanisms involved in cerebral malaria and systemic immunology remain unclear. Experimental models have been established in order to address these issues and develop better therapeutic techniques. As a result, different malaria models have been developed in monkeys, rats and birds with peculiar characteristics. Although none of these models completely reproduces human malaria, some aspects of the disease are similar (Langhorne, 1994; Krettli et al., 2001; De Souza and Riley, 2002; Slater, 2005; Frevert et al., 2008).

Some animal models are considered better suited for cerebral malaria. For example, *Plasmodium knowlesi* and *Plasmodium coatneyi* in cynomolgus monkeys are satisfactory malaria models. Another experimental model used to understand the molecular events that lead to brain pathology is the *Plasmodium berghei* murine model (Martins et al., 2009). Several species are used for murine studies, but only *P. berghei* (ANKA strain) causes cerebral malaria

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doi:10.1016/j.vetpar.2010.04.032

(De Souza and Riley, 2002). Avian malaria was the first model used for the systematic study of this disease; this model dominated studies on the biology of the parasite, immunology and chemotherapeutic research from 1890 to 1940 (Paraense, 1946; Garnham, 1966) and has undergone a recent comeback (Krettli et al., 2001; Permin and Juhl, 2002; Slater, 2005; Williams, 2005; Frevert et al., 2008; Silveira et al., 2009). *Plasmodium gallinaceum* parasite in chickens was described by Brumpt in 1935 and was a versatile model for studying alternative treatments for human malaria (Paraense, 1946; Garnham, 1966; Williams, 2005). Recently, the exo-erytrhocytic forms of *P. gallinaceum* have been described in vascular endothelia of brains with modern localization techniques (Frevert et al., 2008). Therefore, avian malaria is a suitable model for understanding the immune system of chickens, biology of *P. gallinaceum* and malarial diseases.

The aim of the present study was to characterize typical clinical changes, brain histology and nitric oxide production of macrophages in chickens infected with *P. gallinaceum*.

2. Materials and methods

2.1. Parasite and birds

The protozoan *P. gallinaceum*, strain 8A, was kindly provided by Dr. Paulo Pimenta and Dr. Antoniana Krettli from the Renè Rachou Research Center (Oswaldo Cruz Foundation, Minas Gerais, Brazil). The parasite was kept by successive passages in chickens and in its vector (*Anopheles darlingi*).

White leghorn chickens (1-day old) were acquired from commercial establishments in the city of Belém, Pará, Brazil. The birds were kept in cages at the animal facility of the Universidade Federal do Pará, with water and balanced feed *ad libitum*. The birds were infected after 9 days. Three independent experiments with 15 infected and 10 uninfected birds were performed. In all experiments, five birds from the infected group with different parasitemia and five from the control group were sacrificed in parallel with CO_2 for brain histology and macrophage culture. Survival, parasitemia, haematocrit and temperature were determined in all experiments. The results of 30 infected (six chronically) and five uninfected birds are shown together and expressed as mean and standard deviation with minimal and maximal values in parentheses.

2.2. Infection and clinical observations

Chickens were inoculated intravenously with 1×10^6 erythrocytes containing *P. gallinaceum* (Permin and Juhl, 2002). The birds were clinically examined and monitored daily beginning with the 2nd day post-infection (pi) for 15 days or until the day of death. Control experiments showed that inoculation of non-infected blood did not induce changes to clinical signs of the chickens nor nitric oxide production of macrophages. Lack of appetite was evaluated by comparing the amount of eaten feed between infected and non-infected chickens.

2.3. Determination of parasitemia, haematocrit and temperature

Blood samples were obtained from the wing vein every other day, smears were stained with 10% Giemsa for 30 min and observed under a microscope (Silveira et al., 2009). The percentage of parasitemia was estimated counting the number of parasites per 1000 erythrocytes from the border of the smears. For such, erythrocytes in 10 microscopic fields were scored using an immersion lens (100×) and the percentage of infected cells was calculated. Haematocrit values were determined for each bird. Blood was placed in glass capillaries and microcentrifuged (Williams, 1986a). Animal temperature was evaluated daily between 3:00 and 4:00 pm by inserting a thermometer into the cloaca for 3 min (Williams, 1986b).

2.4. Brain histology

Birds with different parasitemia (10, 20, 40, 50 and 60%) were sacrificed; their brains were dissected by craniectomy and fixed in 4% buffered formalin. Samples were routinely processed in alcohol and xylol and embedded in paraffin. Paraffin blocks were cut using a rotational microtome. Sections of 5 μm were stained with hematoxylin–eosin (Egima et al., 2007) and observed under an Axiophot microscope (Carl Zeiss) equipped with a digital system.

2.5. Activation of macrophages derived from blood monocytes and nitrite evaluation

Macrophages derived from monocytes were obtained from the blood following the procedure described by DaMatta et al. (2000). Briefly, blood from heart punctures of uninfected (control) and infected birds with different parasitemia was collected, diluted (1:1) in Dulbecco's modified Eagle's medium (DMEM—Sigma), laid over a Histopaque (Sigma) cushion and centrifuged at $600 \times g$ at 25 °C for 20 min. Leukocytes were collected, washed twice ($500 \times g$, 4 °C, 10 min), adjusted to a density of 2×10^7 cells/mL and 150 μL were seeded on 24-well plates. After adherence, the cells were washed and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Macrophages were cultured at 37 °C in a 5% CO_2 atmosphere for 4 days with wash and medium changed after one and 3 days.

A chicken INF-γ-conditioned medium was obtained by culturing spleen cells with Concanavalin A (Con A). The spleen was mechanically disrupted in DMEM, washed and treated with a lysing buffer. The cells were washed and cultured in DMEM with 10% FBS and 15 μg/mL of Con A. After 48 h, the cells were centrifuged ($500 \times g$, 4 °C, 10 min) and the supernatant was alliquoted and stored at −20 °C.

After 4 days of culture, macrophages were activated by the addition of 25% (v/v) chicken INF-γ-conditioned medium with or without 1 μg/mL of lipopolysaccharide in DMEM supplemented with 10% FBS. After 24 h, the supernatant was analyzed for nitrite content using the Griess reagent (Green et al., 1982).