Short Communication

Immune response in dogs experimentally infected with *Paracoccidioides brasiliensis*


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The aim of the present study was to evaluate the immune response of young dogs experimentally infected with *Paracoccidioides brasiliensis*. Six dogs were infected intravenously with *P. brasiliensis* and one control dog was inoculated with sterile saline. The infected animals were sacrificed in groups of two at 1, 6 and 12 months after infection. During the experimental period, the immune responses of the dogs to the fungus were followed by ELISA (IgM and IgG), by the immunodiffusion test and by the skin test with gp43. After killing the dogs, samples from several organs were submitted to histopathological analysis (H&E and Grocott stains) but the fungus was not observed in any tissue. Attempts to isolate the fungus from these tissue samples were also unsuccessful. All infected dogs, except one, reacted positively to the immunodiffusion and skin tests. All infected dogs showed a humoral immune response to the gp43 antigen detected by ELISA. The IgM and IgG response peaked by the first and second month, respectively. We conclude that young dogs appear to be resistant to the development of paracoccidioidomycosis.

Keywords  dog, fungus, immunity, paracoccidioidomycosis

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The primary infection occurs in the lungs, with dissemination by blood and/or lymphatics to other organs and tissues such as skin, lymph nodes, liver and spleen. PCM most frequently affects rural male workers from Latin America [1]. The *P. brasiliensis* habitat probably is the soil, as is the case for other human pathogenic fungi, although few isolates have been obtained from this source until now [2–4]. The role of other animal species in *P. brasiliensis* ecology is poorly understood [5]. The fungus has only been frequently isolated from the armadillo *Dasypus novemcinctus* [6–9]. The habit of sniffing and digging the soil could expose dogs to *P. brasiliensis* infection. In a recent seroepidemiologic study we have shown high antibody levels to *P. brasiliensis* in dogs from a rural area without clinical evidence of disease [10]. In a previous study, an experimental infection was carried out with 3-month-old puppies and two out four puppies died, presenting extensive granulomatous lesions mainly in the lungs 1 week after *P. brasiliensis* inoculation [11]. The acute evolution of PCM in these animals was probably related to age. Therefore, the aim of the present study was to evaluate the course of experimental PCM in young dogs.
Materials and methods

Experimental animals

Seven mixed-breed dogs from two different litters, weighing 2.5 kg each, were dewormed with Drontal Puppy (Bayer, Rio de Janeiro, Brazil), treated against fleas and ticks with Frontline (Merial, Toulouse, France) and vaccinated (Vanguard HTLP 5/CV-L; Pfizer Animal Health, New York, USA). To prevent P. brasiliensis sensitization before the experimental infection the dogs were followed since birth. The animals were maintained isolated in cages with water and commercial dog food ad libitum. Seven days before infection, when all dogs were 8 months old, the animals were observed for clinical signs and blood samples were collected for serology. Before infection, all animals were healthy and negative for anti-P. brasiliensis gp43 antibodies and a skin test was negative for gp43. The animals were housed in accordance with the Guide for Care and Use of Laboratory Animals, National Research Council, 1996.

Fungus

Paracoccidioides brasiliensis LDR1 isolate (obtained from a chronic PCM patient) [12] was inoculated in guinea pig testicle and reisolated prior to experimental infection in dogs in order to maintain fungal virulence. The fungus was grown on Sabouraud peptone glucose agar (Difco, Detroit, MI, USA) for 7 days at 35°C. The yeast cells were collected and homogenized in sterile saline and their concentration was adjusted to 2.5 × 10⁶ cells/ml.

Experimental infection

Six 8-month-old male dogs were individually infected via the brachial vein with the P. brasiliensis isolate LDR1 (1 × 10⁶ cells in 400 µl sterile saline) and a control 8-month-old female dog was inoculated with 400 µl sterile saline by the same route. The infected dogs were killed in groups of two by intravenous injection of KCl after anaesthesia with acepromazine (0.2 mg/kg) and thiopental (12.5 mg/kg) at 1, 6 and 12 months post inoculation. Samples from lungs, spleen, liver, lymph nodes, skin, kidneys, heart and brain were submitted to histopathological analysis (H&E and Grocott stain), and tissue portions were seeded on Sabouraud peptone glucose agar followed by incubation at 35°C for 40 days. Two dogs were submitted to oral treatment with prednisone tablets (Indústria Química e Farmacêutica Schering-Plough S/A, Rio de Janeiro, Brazil) at month 8 post-infection (Meticorten, 2.2 mg/kg/day during the first week and 6.6 mg/kg/day during the second week) [13].

Clinical observations

Animals were observed daily for systemic signs of disease such as fever, weight loss, alertness, and anorexia.

Collection of blood samples

Blood samples were collected from each animal by brachial vein puncture at days 0, 10, 20, 30 after infection and at monthly intervals thereafter.

P. brasiliensis antigens

The exoantigen was obtained from P. brasiliensis, B-339 isolate, as described by Camargo et al. [14] and gp43 was obtained from the exoantigen by affinity chromatography as described previously [10].

Skin test with gp43

The skin test was performed as described previously [10].

Immunodiffusion test

The immunodiffusion test was performed as described by Camargo et al. [14] using P. brasiliensis exoantigen as reagent.

IgG and IgM anti-gp43 detection in dog sera by ELISA

Polystyrene microtitre plates (Corning Costar, Corning, New York, USA) were coated with gp43 (250 ng per well) in 0.1 mol/l carbonate buffer, pH 9.6. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and the wells were blocked with PBS-T 5% skim milk (PBS-T-M) for 1 h at 37°C. After washing with PBS-T, the serum samples were diluted 1:100 in PBS 1% skim milk (PBS-M) and incubated at 37°C for 1 h. The plates were washed as above and 100 µl of conjugate anti-dog IgG-peroxidase (Sigma, St Louis, MO, USA) or anti-dog IgM-peroxidase (Bethyl, Montgomery, TX, USA) were added to each well. Plates were then incubated at 37°C for 1 h. After washing with PBS-T, substrate (H₂O₂/tetramethylbenzidine) was added, and the reaction was stopped by adding 50 µl of 4 N H₂SO₄. Absorbance was measured at 450 nm with an ELISA reader (Flow Laboratories, McLean, VA, USA). Serum from a dog immunized with P. brasiliensis was used...
as a positive control. The negative control was a pool of sera from urban dogs. Sera with twice or more the absorbance of the negative control were considered positive.

Statistical analyses
The data were analysed by the Student-Newman-Keuls multiple comparison test. The difference was considered significant when $P$ was less than 0.05.

Results and discussion
No evident clinical signs of systemic illness were observed in any of the infected dogs. Two animals (dogs 3 and 5) showed lymph node enlargement 13 days after infection (left submandibular and left popliteal nodes, respectively). Aspirates were collected from these lymph nodes but $P$. brasiliensis was not detected by direct examination or by culture. The two dogs submitted to corticosteroid therapy for 2 weeks showed no sign of disease although a decrease in the intradermal reaction to gp43 was observed in one dog (Table 1).

Positive intradermal reactions to gp43 were observed in 5 of 6 dogs infected with $P$. brasiliensis. The reactions to gp43 ranged from 5 to 12.7 mm in diameter (Table 1). The non-infected dog (control) was negative at all times.

All infected dogs except one were positive to the immunodiffusion test with $P$. brasiliensis exoantigen and positivity was detected 20 days after infection (Table 2). No reaction was observed with serum samples from the non-infected dog.

The infected dogs showed a humoral immune response to gp43, producing IgM (all animals) and IgG (all animals, except one). The antibody response began on day 10 after infection and the peak of IgM and IgG occurred at 20 and 60 days after infection, respectively, with antibodies being detected up to the month 12 (Figs. 1 and 2).

Dogs are in close contact with soil, the presumed habitat of $P$. brasiliensis, and seroepidemiologic studies have shown high positivity in dogs from endemic PCM areas [10,15]. Despite the high infection rates observed in dogs, there is only one report so far of a dog that developed PCM [16]. As little is known about the susceptibility of dogs to PCM, in this study we followed the immune response in dogs experimentally infected with $P$. brasiliensis.

A humoral immune response was detected in all infected animals by ELISA although one dog was negative by the immunodiffusion test, ratifying the higher sensitivity of the enzyme immunoassay, especially for IgM detection that peaked by day 20 after infection, although IgG peaked by the day 60. The decrease in immune response observed at month 12 post infection suggests that the dogs’ immune mechanisms were effective against the fungus, although

### Table 1

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nd = not done (euthanasia).

Non-infected dog (control) was negative at all times.

### Table 2

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= euthanasia.
+ = positive.
– = negative.
d = days.
m = months.

Non-infected dog (control) was negative at all times.
the maintenance of low levels of antibodies could be due to some viable *P. brasiliensis* cells in organs or tissues, stimulating the host immune response. The lower humoral immune response observed in one dog is probably due to individual variability as expected in non isogenic animals.

The detection of a positive reaction to the immunodiffusion test suggests development of PCM since in humans only individuals with the active mycosis are positive to this test. The negative immunodiffusion tests at the fifth and sixth month after infection may have been due to an increase in asymmetric antibody levels, as described for human PCM [17]. Another explanation could be the lower sensitivity of this test, if we consider that these samples were positive by ELISA.

The strong reaction to the gp43 skin test in five infected dogs suggests that these animals developed a cellular immune response to the fungus as previously observed in puppies that survived *P. brasiliensis* infection [11].

Taking into account that high glucocorticoid doses can be associated with severe fungemia [18] two infected dogs were submitted to treatment with prednisone for 2 weeks. These animals showed no sign of disease although a decrease in humoral

**Fig. 1** Levels of IgM anti-gp43 evaluated by ELISA in sera from dogs experimentally infected with *Paracoccidioides brasiliensis* (dogs 1–6) and a non-infected dog (control). The IgM levels in infected animals were significantly different from control (*p* <0.05).

**Fig. 2** Levels of IgG anti-gp43 evaluated by ELISA in sera from dogs experimentally infected with *Paracoccidioides brasiliensis* (dogs 1–6) and a non-infected dog (control). The IgG levels in infected animals, except dog 2, were significantly different from control (*p* <0.05).
and cellular immune response was observed one month after prednisone therapy. The failure of prednisone to induce PCM may have been due to the short time of treatment.

The lack of clinical signs in experimentally infected dogs and the failure to isolate *P. brasiliensis* from tissues suggest that young dogs are resistant to PCM development. These data agree with those reported by Mós and Fava Netto [19] who observed no signs of disease in adult dogs inoculated with *P. brasiliensis*. In contrast, Pereira and Vianna [20] inoculated a dog with pus from a PCM patient and the animal died 3 weeks after infection. This disagreement may result from differences in the virulence of *P. brasiliensis* isolates, size of the inoculum or genetic differences among animals.

Although in nature *P. brasiliensis* infection probably occurs by propagules inhalation, in this study the intravenous route was chosen because in a previous work with puppies this route was effective, causing disease in two out of four animals [11].

The susceptibility to PCM development shown by puppies in our previous study and the resistance of young dogs observed in the present study suggest that age is an important risk factor in canine paracoccidioidomycosis.

**Acknowledgements**

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**References**