ACTIVATION OF THE ALTERNATIVE COMPLEMENT PATHWAY IN CANINE NORMAL SERUM BY PARACOCCIDIOIDES BRASILIENSIS

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ABSTRACT

The dimorphic fungus Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis, a human granulomatous disease. Recently the first case of natural disease in dogs was reported. The complement system is an important effector component of humoral immunity against infectious agents. Therefore, the aim of this study was to evaluate the activation of the dog alternative complement pathway by P. brasiliensis. Initially, the ability of erythrocytes of guinea pig, rabbit, sheep, chicken and swine to activate the dog alternative pathway was evaluated. The guinea pig erythrocytes showed the greatest capacity to activate dog alternative pathway. The alternative (AH50) hemolytic activity was evaluated in 27 serum samples from healthy dogs and the mean values were 87.2 AH50/ml. No significant differences were observed in relation to sex and age. The alternative pathway activation by P. brasiliensis was higher in serum samples from adult dogs when compared to puppies and aged dogs (p ≤ 0.05). This is the first report of dog alternative complement pathway activation by P. brasiliensis and suggests that it may play a protective role in canine paracoccidioidomycosis.

Key words: Paracoccidioidomycosis, dog, complement

INTRODUCTION

Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis, a systemic mycosis prevalent in Latin America. The fungus grows as mycelia when cultured at 25ºC or as yeast when cultured at 37ºC or at the host. Male agricultural workers are most affected by the disease and the infection probably occurs by inhalation of fungus propagules (3,5).

Epidemiological studies have demonstrated that infection by P. brasiliensis occurs in domestic and in wild animals (6,7,8,11,15,18,22,24,25). A high frequency of infection was observed in dogs from areas endemic for paracoccidioidomycosis in Brazil (18,24), although to date, only two cases of natural disease have been reported (4,12).

The dog habit of sniffing and digging the soil, the probable P. brasiliensis habitat, may increase the risk of infection. The low frequency of natural disease in dogs may be due to failure in diagnosis or to natural resistance.

The complement system is an important immune effector mechanism against infectious agents (14) and the activation of the complement has been reported in human paracoccidioidomycosis and other mycosis (16,17,21,23,27). Therefore, the aim of this study was to evaluate the activation of the canine alternative complement pathway by P. brasiliensis.

MATERIALS AND METHODS

Serum samples

A total of 27 serum samples were obtained from apparently healthy dogs with ages ranging from 1 to 15 years. The blood was collected by venous puncture in sterile glass tube and centrifuged within 1 hour of collection. The serum samples were stored at -80ºC until use. All the samples were negative to IgM and IgG against P. brasiliensis gp43 by the ELISA test, performed as previously described (9).
**P. brasiliensis**

The *P. brasiliensis* isolate B-339 was cultivated on Sabouraud Dextrose Agar for seven days at 35°C. The yeast cells were collected in PBS and killed by autoclave (121°C for 15 min.).

**Activation of the dog alternative complement pathway by erythrocytes from different animal species**

Blood samples were collected from chicken, guinea pig, rabbit, sheep and swine and maintained in Alsever’s solution at 4°C until use. Erythrocyte suspensions (2.8%) in borate buffer saline containing 0.5 mM Mg²⁺ and 0.03 M EGTA (BBS-Mg-EGTA) were incubated with dog serum (diluted 1:5) for 30 min. at 37°C. After centrifugation (2,000xg) the supernatants were analyzed in a Microplate Reader at 492 nm. The percentage of lysis was calculated in relation to the 100% lysis control (erythrocyte suspension and distilled water).

**Evaluation of alternative complement pathway activity in dog serum samples and alternative complement pathway activation by *P. brasiliensis***

The alternative complement pathway activity (AH50) was evaluated by a micromethod (20) using guinea pig erythrocytes. The serum samples diluted 1:10 in BBS-Mg-EGTA were added to 1.5 mL microcentrifuge tubes at volumes of 40, 60, 80 and 100 µl and the final volume was completed with BBS-Mg-EGTA. The 2.8% suspension of guinea pig erythrocytes was added (100 µl) and after incubation for 30 min. at 37°C, the tubes were centrifuged (2,000xg) and the supernatants were analyzed in a Microplate reader at 492 nm. The percentage of lysis was calculated in relation to 100% lysis control (erythrocyte suspension and distilled water) and plotted in a graph for AH50 determination.

The evaluation of alternative complement pathway activation by *P. brasiliensis* yeast cells was performed by determination of AH50, as described above, in serum samples preincubated with inactivated *P. brasiliensis* yeast cells (2 x 10⁵ cells in BBS-Mg-EGTA), for 30 min. at 37°C in an orbital shaker (100 rpm). As control, the serum samples were preincubated for the same time with BBS-Mg-EGTA only. The alternative complement activation was determined as follows: AH50/ml in serum sample - AH50/ml in serum sample preincubated with *P. brasiliensis*.

**Statistical analysis**

The data were analyzed by ANOVA and the Tukey-Kramer test (STATISTIC 6.0). The difference was considered significant when P ≤ 0.05.

**RESULTS AND DISCUSSION**

As a preliminary study, the most adequate erythrocyte for canine alternative pathway activation was evaluated. Erythrocytes from sheep, chicken, rabbit, swine and guinea pigs were assayed. Guinea pig erythrocytes showed the highest efficiency for alternative pathway activation and hence they were used for AH50 determination (Fig. 1).

Tanaka *et al.* (26) evaluated the activation of dog alternative complement pathway by erythrocytes from different animal species and the swine erythrocytes showed higher activation efficiency than guinea pig and rabbit erythrocytes (26). The disagreement observed was probably due to differences between the methodologies used in both studies. Taking into account that the guinea pig erythrocyte is more easily available than swine erythrocytes, this result makes the alternative complement evaluation in dog serum more suitable.

The alternative complement activity evaluated in serum samples showed an average of 87.2 ± 51.9 AH50/ml and no significant difference was observed in relation to gender or age (Table 1).

| Animal       | Erythrocyte lysis (%) |
|--------------|-----------------------|
| guinea pig   | a                     |
| rabbit       | b                     |
| swine        | b                     |
| sheep        | b                     |
| chicken      | c                     |

**Figure 1.** Activation of the dog alternative complement pathway by erythrocytes from different animal species. Bars with different letters indicate statistically difference (P ≤ 0.05).

**Table 1.** Alternative complement activity in dogs’ serum samples according to sex and age.

|                        | AH50/ml     |
|------------------------|-------------|
| **Sex**                |             |
| Male (n = 16)          | 80.9 ± 53.6 |
| Female (n = 11)        | 96.3 ± 50.6 |
| **Age (years)**        |             |
| < 1 (n = 8)            | 67.9 ± 52.8 |
| 1 - 8 (n = 10)         | 88.8 ± 54.9 |
| 9 - 15 (n = 9)         | 101.1 ± 52.0 |
| **Total** (n = 27)     | 87.2 ± 51.9 |

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Taking into account that the complement is an important effector mechanism against fungal infection (12) in this study the activation of the dog alternative complement pathway by *P. brasiliensis* was evaluated. The alternative pathway activation in dogs aged between 1 to 8 years was significantly higher than animals up to 1 year and animals over 9 years old (Fig. 2).

This is the first report that *P. brasiliensis* activates canine alternative complement pathway and suggests that it can play a protective role.

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RESUMO

Ativação da via alternativa do complemento em soro de cão normal por *Paracoccidioides brasiliensis*

O fungo dimórfico *Paracoccidioides brasiliensis* é o agente etiológico da paracoccidioidomicose, uma doença granulomatosa humana. Recentemente, foi relatado o primeiro caso da doença natural em cães. O sistema complemento é um importante componente efetor da imunidade humoral contra agentes infecciosos. Portanto, o objetivo deste trabalho foi avaliar a ativação da via alternativa do complemento canino pelo *P. brasiliensis*. Inicialmente, foi avaliada a capacidade de eritrócitos de cobaia, coelho, carneiro, galinha e suíno ativarem a via alternativa do complemento canino. Os eritrócitos de cobaia apresentam maior capacidade de ativar a via alternativa do complemento canino. A atividade hemolítica da via alternativa (AH50) foi avaliada em 27 amostras de soro de cães sádáveis e os valores médios observados foram de 87,2 AH50/ml. Não foi observada diferença significativa ao sexo e idade. A ativação da via alternativa pelo *P. brasiliensis* foi maior nas amostras de soro de cães adultos quando comparada aos cães filhotes e idosos (p ≤ 0.05). Este é o primeiro relato da ativação da via alternativa do complemento canino pelo fungo *P. brasiliensis* e sugere que pode ter um papel protetor na paracoccidioidomicose canina.

Palavras-chave: Paracoccidioidomicose, cão, complemento

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