

## NATURAL ANTIBODIES IN PARACOCCIDIOIDOMYCOSIS

Carmelinda S. Unterkircher<sup>1</sup>; Mariella V.P. Leão<sup>2\*</sup>; Maria Heloisa S.L. Blotta<sup>3</sup>; Zoilo Pires de Camargo<sup>4</sup>

<sup>1</sup>Departamento de Biociências e Diagnóstico Bucal, Faculdade de Odontologia, Universidade Estadual Paulista, São José dos Campos, SP, Brasil; <sup>2</sup>Departamento de Medicina, Universidade de Taubaté, Taubaté, SP, Brasil; <sup>3</sup>Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brasil; <sup>4</sup>Disciplina de Biologia Celular, Escola Paulista de Medicina, São Paulo, SP, Brasil.

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### ABSTRACT

Recent attention has been focused on the natural antibodies as a component of natural immunity and as integral part of the idiotypic network. However, their functional role in different infections has rarely been studied. This work was undertaken to investigate the presence of natural antibodies in paracoccidioidomycosis (PCM). In addition, we analyzed anti-*P. brasiliensis* antibodies and their distribution in IgG subclasses in order to acquire better knowledge about the humoral immune response in this mycosis. Our findings show that the natural antibody response is not very much increased in PCM when compared with other parasite infections and this response is restricted to a few specificities, suggesting that *P. brasiliensis* moderately triggers CD5+ B cells. The anti-actin antibody was the main antibody specificity found in PCM. Specific antibodies to *P. brasiliensis* were mainly found in the IgG1 subclass in chronic patients of PCM.

**Key words:** natural antibodies, *Paracoccidioides brasiliensis*, paracoccidioidomycosis

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### INTRODUCTION

Paracoccidioidomycosis (PCM) is a chronic granulomatous disease, caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The natural infection starts by the inhalation of airborne mycelial propagules of its saprobic phase that quickly converts to the yeast form in tissues, producing a wide clinical spectrum of disease, ranging from localized pulmonary lesions to disseminated disease (18). PCM is the most important mycosis found in Latin America where it is frequently diagnosed in rural workers (11). It has been suggested that the pathological process is a result of reactivation of quiescent lesions acquired many years before (11).

The biological heterogeneity of *P. brasiliensis* strains and the host genetic background may conceivably contribute to the varied clinical expressions of the disease (6,12).

The cellular immune response is usually suppressed, to different degrees in PCM, whereas the humoral immune response

is not impaired and a high level of specific antibodies may be detected by several techniques (4,7,9,10,22). Other alterations in the immune response include hypergammaglobulinemia, IgE hyperproduction and eosinophilia (1).

In normal individuals CD5+ B cells are committed to secrete natural antibodies endowed with multiple antigen-binding specificities including to self-constituents and foreign antigens (2,8). Natural antibody levels increase transiently in various infectious diseases, returning to normal after recovery from infection (14,19,21). These antibodies have also been found in many diseases, associated with immune complexes, in humans and mice (2). It was demonstrated that non-mutated germ-line genes encode natural antibodies and that these participate in immune regulation through a large number of interaction (3). Moreover, it seems that these autoantibodies operate in tandem with  $\gamma\delta$  T cells as components of natural immunity protecting mucosal surfaces against many invaders (15).

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\*Correspondent author. Mailing address: Av. Tiradentes, 500, Bom Conselho, 12030-180, Taubaté, SP, Brasil. Tel.: (+5512) 39218166. E-mail: leaomari@hotmail.com

In PCM knowledge of the host-parasite relationship is still incomplete and no information can be found in the literature about the natural antibody response during the evolution of the disease. The observations herein reported suggest that in PCM different sorts of B cell clones are triggered by infection. The clones include some secreting autoantibodies, particularly anti-actin antibodies.

## MATERIALS AND METHODS

### Population studied

Serum samples were obtained from the Clinics Hospital, UNICAMP, Campinas, São Paulo, Brazil. The patients were selected by clinical and laboratorial criterions that included serodiagnosis by immunodiffusion with specific antigen of *P. brasiliensis* and fungus isolation in same cases (7). Histoplasmosis was investigated by sorological test with specific antigen. The patients (n=40) included in this study were classified as chronic (CF, n=30) and juvenile (JF, n=10) forms of disease using a clinical criterion. 30 males composed the chronic group and their mean age was 52 years. Five females and 5 males constituted the juvenile group. The average age of the group was 18.5 years. The sample sera were taken before antimycotic therapy. The majority of the patients were from Caucasian origin. Most of them were rural workers from the region of Campinas. In addition, 20 serum samples were obtained from healthy individuals from the Clinics Hospital Blood Bank and they were negative for *P. brasiliensis*, hepatitis virus, human immunodeficiency virus, *Treponema pallidum* and *Trypanosoma cruzi*. All serum specimens were divided into aliquots and kept frozen at -20°C.

### Fungal strain

*P. brasiliensis* B-339 was obtained from A. Restrepo, Corporation Investigaciones Biologicas, Medellin, Colombia and has been maintained by frequent subculture (every third day) on Sabouraud dextrose agar (60 g neopeptone, 36 g glucose, 1.5 g agar) at 25°C. The fungus was converted to the yeast form on modified Sabouraud dextrose agar containing 0.01% (w/v) thiamine and 0.14% (w/v) asparagine at 35°C.

### Antigen preparation

*P. brasiliensis* yeast form ( $2 \times 10^6$  cells) was grown in 1,800 ml of neopeptone medium (60 g neopeptone, 36 g glucose, 0.18 g thiamine, 2.5 g asparagine), divided in three Fernbach flasks. After incubation (7 days, 35°C) on an orbital shaker at 50 rpm, it was killed with Merthiolate (0.2g/L) (7). The cells were washed three times with cold buffer (Tris-HCl 50 mM, pH 8.0, EDTA 2 mM, PMSF 5 mM) and disrupted mechanically with glass beads by agitation for 18 hours at 4°C. PMSF was added three times during the lysis cycle. The suspension was subsequently centrifuged at 13,620-x g for 30 min at 4°C, to remove fragments

of the cellular wall, and the supernatant was lyophilized and stored at -20°C. Protein measurements were made by the Bradford's method (5).

### Enzyme immunoassay (EIA)

Polystyrene flat-bottomed plates (Hemobag) were coated with actin, tubulin, myosin, TNP-OVA, myoglobin and *P. brasiliensis* cytoplasmic antigen (5 µg/ml) and DNA (10 µg/ml) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, for 2 h at 37°C and stored at 4°C. Before the assay, the free binding sites of coated plates were blocked with 0.5% gelatin in phosphate-buffered saline (PBS) for 1h at 37°C. Following this, sera were diluted (1/50) in PBS containing 0.5% gelatin and 0.1% Tween 20 (PBS-T-G) and placed in duplicate wells (50 µl) and the plates were incubated for 90 min at 37°C and overnight at 4°C. After washing in PBS with Tween 20 (PBS-T), the plates were incubated for 90 min at 37°C with specific antibodies (1 µg/ml) against  $\gamma$ ,  $\alpha$  and  $\mu$  chain, coupled with  $\beta$  galactosidase (Tago, Burlingame, CA, USA) or peroxidase (Sigma) (1 µg/ml) prepared in PBS-T-G. The plates were washed five times in PBS-T and the enzyme specific substrate added. Optical density was determined at 414 nm or 492 nm in an EIA reader (BIO RAD model 3550).

### IgG subclass analysis

Subclasses were determined in twenty sera by EIA using subclass-specific monoclonal antibodies. NL16 (anti-IgG1), GOM2 (anti-IgG2), 2G4 (anti-IgG3) and RD4 (anti-IgG4) were purchased from Oxoid (Basingstoke, Hants, England) and were assayed using peroxidase anti-mouse conjugate absorbed against human IgG (Sigma Chemical, Co). As control, we have used 20 normal sera tested at the same time.

### Expression of results and statistical analysis

The results were expressed in percentage values, considering 100% to be the arithmetic mean of reactivity obtained in normal sera (n=20) for each antigen. Statistical significance was determined by unpaired Student's t-test ( $p < 0.01$ ). The results of the subclass-specific antibody analysis were expressed as absorbance at 492 nm. Control sera displayed a very lower reactivity in this assay.

## RESULTS AND DISCUSSION

Several infectious diseases caused by parasites, bacteria and viruses are associated with the induction of polyspecific antibodies that are able to bind several self and foreign antigens (18,22). B cells bearing the CD5+ surface marker mainly synthesize the natural antibodies (15,17). This set of cells may be directly stimulated by B cell mitogen, such as bacterial lipopolysaccharide (LPS) or be induced by an antigen-driven process (15).

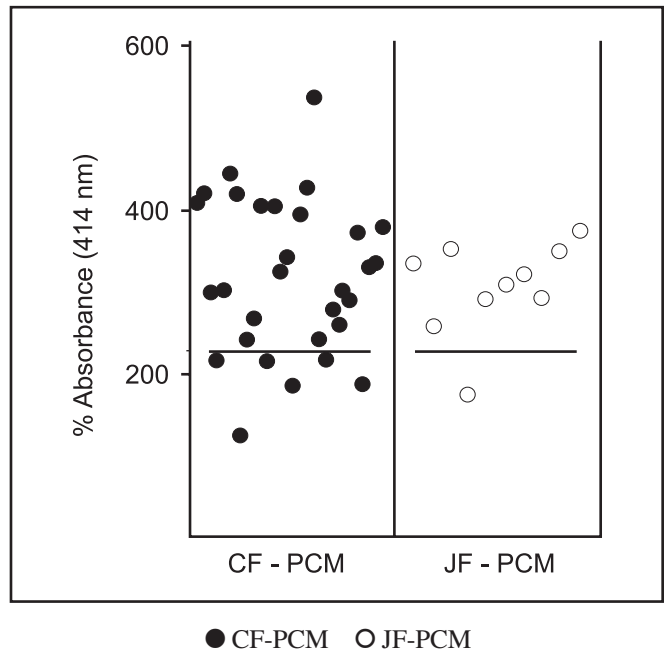
Natural antibody level was analyzed in thirty CF-PCM sera and ten JF-PCM (Fig. 2). Some chronic patients had increased

levels of antibodies against actin (46%), TNP (20%), DNA (16%), tubulin (16%) and myosin (13%) in the IgG isotype. In the juvenile form of disease a significant level of anti-actin IgG antibodies (mean=242, SD=122) could be observed, but no increase was detected for other antigens tested. Rises in IgM and IgA antibodies could not be demonstrated. A significant decrease in anti-TNP and anti-DNA IgM antibodies levels ( $p < 0.01$ ) was found in PCM sera, when compared with their respective controls (Fig. 3), although the exact meaning of this finding is unclear. It may be suggest either to the presence of these antibodies in immune complexes or a suppressive control by T cells on B cells secreting anti-TNP and anti-DNA antibodies.

The levels of specific IgG antibodies to *P. brasiliensis* were considerably increased in chronic and juvenile forms of PCM ( $p < 0.01$ ) (Fig. 1). The antibody response to *P. brasiliensis* was less variable in the juvenile form (mean=303, SD=57) than in the chronic form (mean=331, SD=106). The IgG subclasses were studied in patients with the chronic form of disease (Fig. 4). Specific antibodies were largely restricted to IgG1. Five patients showed raised IgG2 level. Absorbance over 0.3 O.D. in IgG3 was only seen in one patient. Detectable IgG4 level was found in 6 patients and four of them had also high IgG1 levels.

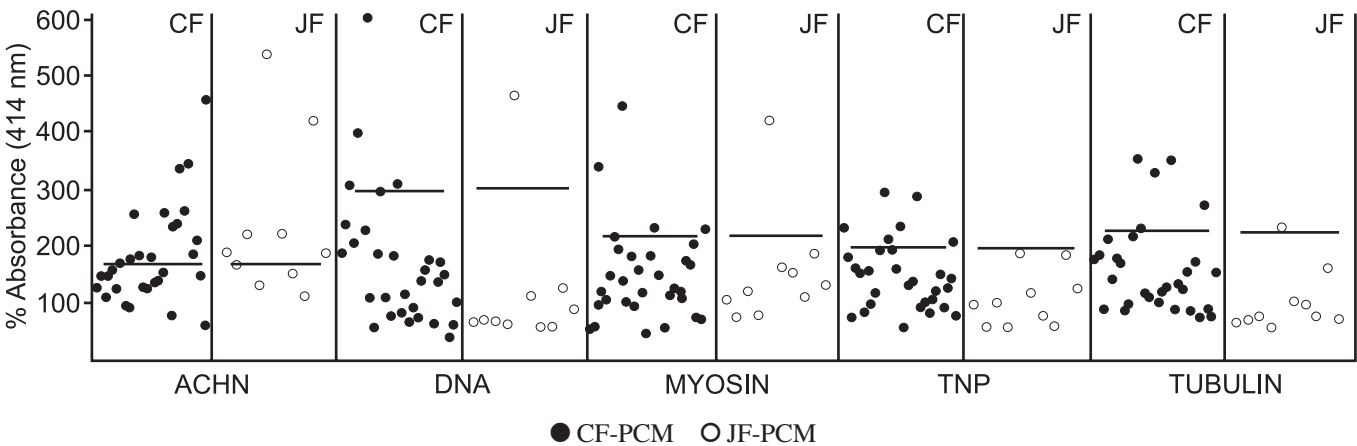
No correlation could be demonstrated between natural and specific antibodies measured by EIA. Analysis of the natural antibody response revealed significant B cell activation in CF-PCM and JF-PCM, especially to anti-actin clones. However, these increases were discrete when compared with the rises seen in parasite infections (20,22).

Our findings suggest that in PCM the natural antibody response vary according to the clinical forms and perhaps to the severity of the disease. CF-PCM sera had high levels of specific antibodies, and a moderate level of natural antibodies. The specific antibodies were mainly concentrated in the IgG1

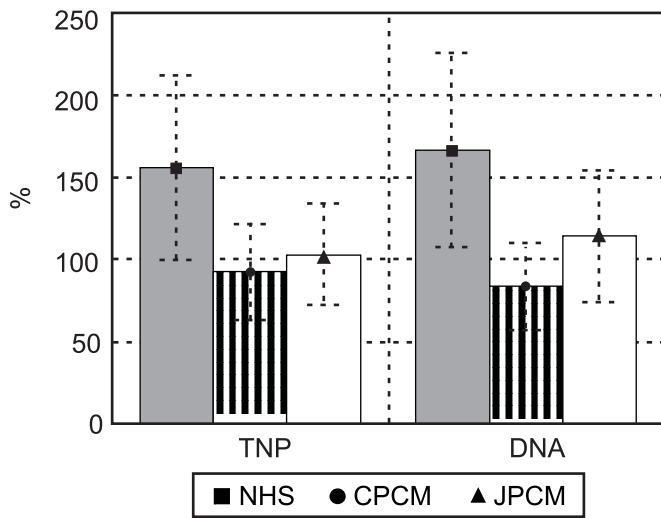


**Figure 1.** Anti-*Pbrasilensis* IgG antibodies in CF-PCM (n=30) and JF-PCM (n=10) sera. Results are expressed as percent absorbance considering as 100% the arithmetic mean of the controls (n=20). Horizontal lines represent the arithmetic mean of the normal sera plus 2 SD.

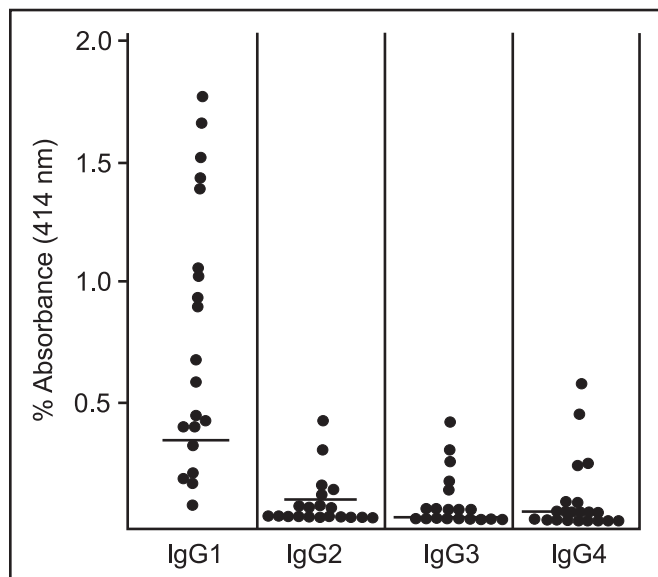
subclass, although some CF-PCM sera displayed a detectable level of IgG4 which may represent a prolonged exposure to antigens or it may reflect the pattern of cytokine secretion by Th2 helper cells (21,22). It had already been demonstrated that susceptibility to *P. brasiliensis* is associated with Th2 response



**Figure 2.** Natural antibodies IgG in CF-PCM (n=30) and JF-PCM (n=10). Results are expressed as percent absorbance considering as 100% the arithmetic mean of the controls (n=20). Horizontal lines represent the arithmetic mean of the normal sera plus 2 SD.



**Figure 3.** Levels of anti-TNP and anti-DNA IgM in NHS (dark), CF-PCM (striped) and JF-PCM (bright). The antibodies levels were expressed as percent absorbance (414 nm). The human normal sera consistently displayed higher anti-TNP and anti-DNA IgM levels (around 150%).



**Figure 4.** Specific IgG subclass analysis in chronic patients of PCM (n=20). Sera were analyzed by EIA using monoclonal antibodies to human IgG subclass and peroxidase labeled anti-mouse immunoglobulins. The bar represent the arithmetic mean of the normal sera (n=20).

(13). Concerning specific antibodies of other immunoglobulin isotypes unchanged IgM and IgA levels were found in PCM sera. These results differ from previous reports where anti-

*P.brasiliensis* IgM was detected by indirect immunofluorescence technique and by Western blot assay (4,11). These conflicting data may be due either to the antigenic preparation used herein.

The finding of antibody to actin in JF-PCM (50%) and CF-PCM (46%) suggests that this antibody specificity may be considered a marker of polyclonal B cell activation in PCM. So, it could be inferred that the activation of B cells, early in the infection might influence T helper cell development. In mice, it has been showed that B1 cells constitutively express IL10, a potent regulator of immune function (16). This subset of B cells could play a role in the alveolar sites binding *P. brasiliensis* carbohydrate antigens and it may influence T cell polarization in the early phase of the infection.

No report was found in the literature on natural antibody levels in PCM. The pattern herein described needs to be confirmed in different clinical forms of PCM.

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#### RESUMO

##### Anticorpos naturais na paracoccidioidomicose

Recente atenção tem sido dada aos anticorpos naturais como componentes da imunidade natural e como parte integrante da rede idiopática. Todavia, seu papel funcional em diferentes infecções tem, raramente, sido estudado. O objetivo deste trabalho foi investigar a presença de anticorpos naturais na paracoccidioidomicose (PCM). Em adição, analisamos os anticorpos específicos anti-*P. brasiliensis* e sua distribuição em subclasses a fim de adquirir mais conhecimento sobre a resposta imune humoral nesta micose. Nossos achados mostram que a resposta de anticorpos naturais não é acentuada na PCM quando comparada com outras infecções por parasitas e, é restrita a poucas especificidades, sugerindo que o *P. brasiliensis* estimula moderadamente as células B CD5+. O anticorpo anti-actina foi a principal especificidade encontrada na PCM. Os anticorpos específicos para *P. brasiliensis*, nos pacientes crônicos, eram, principalmente, da subclasse IgG1.

**Palavras-chave:** anticorpos naturais, *Paracoccidioides brasiliensis*, paracoccidioidomicose

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