HIGH MOLECULAR WEIGHT ANTIGENIC COMPONENTS OF PARACOCCIDIOIDES BRASILIENSIS: PARTIAL CHARACTERIZATION AND IMPLICATIONS FOR TH1 RESPONSE

https://doi.org/10.56238/sevened2024.039-009

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ABSTRACT

Paracoccidioidomycosis (PCM) is a chronic infection caused by the fungi Paracoccidioides brasiliensis or P. lutzii, whose host defense is mediated by Th1 lymphocytes. This study aimed to purify and evaluate the effect of high molecular weight (hMM) components of P. brasiliensis on the cellular immune response. The soluble antigens of the fungus (Pb18) were submitted to Sephadex G-200 column chromatography followed by HPLC, obtaining the F17 fraction (~380 kDa) and its F17-IV subfraction (~70 kDa), both reactive to sera from patients with PCM. The fractions were tested in vitro with the splenic cells of infected mice, resulting in lymphoproliferative response and increased levels of INF-γ and IL-10, without IL-4 alteration. These findings indicate the activation of the Th1 immune response and suggest that the ~380 kDa fraction can degrade generating active subcomponents of ~70 kDa. The ~70 kDa subcomponent appears to be distinct from the gp70 described in the literature, given its ability to induce INF-γ. The results point to the immunogenic potential of these components, highlighting their relevance for the development of vaccines or therapies against PCM.

Keywords: Cytokines, Fungus, INF-γ, Paracoccidioidomycosis, Therapy, Vaccine.

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INTRODUCTION

Paracoccidioidomycosis (PCM) is a chronic infectious disease caused by the fungus *Paracoccidioides brasiliensis* and the new species *Paracoccidioides lutzii*. This mycosis is endemic in Latin America, with a higher prevalence in Brazil, mainly affecting rural workers (San-Blas, 1993, Shikanai-Yassuda et. al., 2017). The fungus has two distinct morphological forms, depending on temperature: a yeast-like form, present in host tissues or when cultured at 35-37°C (Queiroz-Telles, 1994), and a mycelian form at room temperature. This morphological transition is reversible and occurs in response to temperature variation, being regulated by enzymes that modify the synthesis of glucans in the cell wall (San-Blas, 1982).

The fungus P. brasiliensis *or* P. lutzii *is presumed* to inhabit the soil in the mycelial form, and infection occurs by inhalation of fungal propagules. Exposure to the fungus can result in an asymptomatic infection or the development of the disease. In most cases, a primary lung complex is formed that regresses spontaneously with the establishment of specific immunity. However, the fungus can remain in a state of latency for years in residual pulmonary or extrapulmonary lesions. In progressive and disseminated forms, the fungus can reach lungs, mucous membranes, lymph nodes, skin, adrenal glands, and other organs or systems (Londero & Del Negro, 1986; Padilha-Gonçalves, 1985, Shikanai-Yassuda et. al., 2017, Cezar-dos-Santos et al., 2020).

Based on the clinical and natural evolution of the disease, PCM is classified into four main forms: **Infection:** initial phase, usually asymptomatic; **Acute or subacute (juvenile) form:** rarer and more severe, with involvement of lymph nodes, spleen and liver; **Chronic form (adult):** more common, which can vary from localized (unifocal) to disseminated (multifocal), depending on the efficiency of immunity; **Residual form (sequelae):** characterized by permanent lesions resulting from a previous infection (Franco et al., 1987, Shikanai-Yassuda et. al., 2017).

The immune profile also varies according to the form of the disease: **Chronic-unifocal form:** Predominance of T-helper cell response 1 (Th1); **Acute or chronic-disseminated form (multifocal):** Predominance of T-helper 2 (Th2) response, characterized by a higher production of immunoglobulins G (IgG) and the release of specific cytokines (Singer-Vermes et al., 1993; Baida et al., 1999; Cezar-dos-Santos et al., 2020).

Cellular immunity plays a key role in defending against the fungus (Burger et al., 1996). Patients with disseminated infection often have a negative intradermal paracoccidioidin test and poorly defined granulomatous reactions (Montenegro & Franco, 1994). On the other hand, humoral immunity is not considered protective, as the

exacerbated production of antibodies is associated with the severity of the disease (Singer-Vermes et al., 1993; Mello et al., 2002; Cezar-dos-Santos et al., 2020).

Several glycoproteins and other components of the fungus are related to its pathogenicity and immune response. Among the main components, the glycoproteins of 27-kDa (gp27), 43-kDa (gp43), 55-kDa (gp55), 70-kDa (gp70) and 87-kDa (gp87) stand out. High molecular weight glycoconjugates (hMM) and glycolipids obtained by different extraction methods are also present (Puccia et al., 1986; Blotta & Camargo, 1993; Toledo et al., 1995; Ortiz et al., 1998; Salina et al., 1998, Marquez et al., 2005). The high-MM polydisperse fraction, rich in carbohydrate and with heterogenous electrophoretic migration, was first described by Puccia et al. (1986) analyzing *P. brasiliensis* B339 antigen. This fraction was also detected in *P. brasiliensis* Pb18 and in 20 clinical isolates (Fredrich et al., 2010) and in the new species *P. lutzii* – LDR2 (Assolini et al., 2021), suggesting the constant presence of this component in *Paracoccidioides spp*.

These components play a crucial role in the immunomodulation of PCM and can influence both host defense and fungal survival. Research indicates that the immune response to high molecular weight (hMM) components is associated with the clinical evolution of PCM. Marquez et al. (2005) observed that patients with chronic PCM had high levels of IgG, but not of IgE, against the 380 kDa component of *P. brasiliensis,* suggesting that it does not induce a Th2 response.

In the experimental context, Pavanelli et al. (2007) evaluated the effect of the high molecular weight fraction of the fungus in Balb/c mice and evidenced the promotion of protective immunity.

This work partially characterized the high molecular weight (hMM) components of the fungus *P. brasiliensis* and analyzed its immunomodulatory action. The findings reinforce the importance of immunogenic components in the development of vaccine or therapeutic strategies for PCM.

MATERIAL AND METHODS

OBTAINING *CELL-FREE ANTIGEN* (CFA PB18)

The cell-free *antigen* was obtained from *P. brasiliensis* cepa 18 (Pb18), cultured on Sabouraud agar for 5 days at 35ºC, according to Camargo et al. (1991) with some modifications. After 5-day cultivation, the collected fungal mass was transferred to a tube containing buffered phosphate-saline solution (0.15M PBS and pH 7.4), with 2.5 mM PMSF protease inhibitor (Sigma) and 0.02% thimerosol solution. It was homogenized in a vortex

for 15 minutes and then centrifuged at 13,600 x *g* at 4ºC. The protein concentration was determined by the Folin technique (Lowry et al., 1951).

SEPHADEX G-200 COLUMN CHROMATOGRAPHY

A 9 ml sample of CFA Pb18 (4 mg/ml) was applied to Sephadex G-200 column followed by elution in PBS 0.15M pH 7.4. The fractions obtained by the automatic fraction collector were analyzed in a spectrophotometer (Spectrum SP-2000UV) at 280 nm.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A 100 µL sample of the F17 fraction (Sephadex G-200 column) was refracted in an HPLC column (Varian Pro Star) in a semi-analytical column C18. Gradient buffer was used: 50% ammonium acetate buffer (Merck, Germany), 300 mM pH 7.0, and 50% bidistilled water (Milli Q). The pressure used was 1410 at 97 bars, 2 ml/min. The concentrations of subfraction proteins were measured by the Folin technique and stored at -80ºC.

DOTBLOTTING OF SUBFRACTIONS OBTAINED BY FRACTIONATION OF F-17 BY HPLC COLUMN

The 2 µL samples of hMM fractions and subfractions were pipetted on a nitrocellulose membrane (Gibco Invitrogen Corporation, Long Island, New York, USA). After blockade with the blocking buffer, the membranes were washed and incubated with a pool of sera from PCM patients (biological bank of the Laboratory of Applied Immunology, CCB, UEL/CONEP268, process 33455.2011.06)), diluted 1:40, for 2 h at 37ºC. After further washing, the membrane was incubated with anti-human IgG peroxidase conjugate (Sigma 8775) for 1:30 h at 37ºC. The reaction was evidenced with diaminobenzidine (DAB - Sigma) solution and hydrogen peroxide in 0.15M PBS solution.

IMMUNOBLOTTING

Initially, the samples of CFA Pb18, fraction F17 and sub-fraction of hMM-HPLC (F17-IV) and MM protein standard of 10-200 kDa (Invitrogen 1078-010), were submitted to gel electrophoresis with a gradient of 5-15%. After the run, in 1M tris glycine buffer (Pharmacia Biotech –Sweden and Sigma Co., Germany), pH 8.2 to 100 V, the samples were transferred to the nitrocellulose membrane (Gibco Invitrogen Corporation, Long Island, New York, USA) in tris-HCl-methanol buffer at 23 V for 18 hours and 100 V for 1 h. The membrane was blocked with blocking buffer, for 1 h at room temperature, followed by washes and incubation with a pool of serums from patients with PCM dilute 1:40 (2 h at 37ºC. After

further washes, 1:3000 dilute human anti-IgG peroxidase conjugate (Sigma A-8775, Sigma Chemical Co., St. Louis, MO, USA) was added for 1:30 h at 37ºC. The membrane was washed again, which was revealed with DAB (diaminobenzidine-Sigma Co., USA) in 0.15M PBS and hydrogen peroxide.

INFECTION OF BALB/C MICE

Ten male Balb/c mice of 4 to 6 weeks were infected intravenously with 1x106 cells/ml of *P. brasiliensis* (Pb18) and as a control 6 mice were inoculated with 0.15M PBS solution (sterile) and euthanized after 28 days of infection. The mice were handled according to the rules and authorization of the Ethics Committee on Animal Experimentation of the State University of Londrina (CEEA nº 67/08).

EVALUATION OF LYMPHOPROLIFERATIVE RESPONSE

Spleen cell suspension samples from mice infected with *P. brasiliensis* Pb18 for 28 days or not infected were treated with 0.14M ammonium chloride buffer pH 7.2 for red blood cell lysis, centrifuged and washed with sterile PBS. The cells obtained were resuspended in complete RPMI medium (Sigma Co., USA) (L-glutamine, Hepes, garamycin and 10% fetal bovine serum) at a concentration of 1x106 cells/ml, adding: F17 (100 µg/ml), or F17-IV (50 µg/ml) or exoantigen (50 µg/ml) with 100 µL of cell suspension, incubating for 120 hours. Eighteen hours before the cells were harvested, 3.5 µL of tritiated thymidine was added. The stimulation index of lymphoproliferative responses was calculated by releasing radioactivity incorporated into scintillation liquid, counted in a Beckmann LS 6.800 scintillator device. The proliferation index was calculated by the mean reading of the triplicates of the lymphoproliferative response to antigen, in count per minute (cpm), divided by the mean reading of the triplicates of the lymphoproliferative response in the absence of antigen.

LYMPHOCYTE CULTURE FOR EVALUATION OF CYTOKINE LEVELS

The culture was performed as described in the previous item, but with an incubation time of 72 hours. Supernatant samples were collected and stored at -20ºC for further analysis. Anti-IL-10, anti-IL-4 and anti-INF-γ antibodies at a concentration of 3 µg/ml (50 µL) were diluted in 0.06M bicarbonate buffer pH 9.6, added to immunoplates (TPP, Switzerland) and incubated for 18 hours at 4ºC in a humid chamber. The plates were washed with washing buffer, followed by blockade with 100 µL of PBS buffer containing 10% fetal bovine serum with pH 7.0; 0.05% of tween 20 and molico milk 5%. After incubation for 1 hour at

room temperature, 50 µL/orifice of IL-10, IL-4 and INF-γ standards were washed and added, starting with a serial dilution of 2ng/ml in PBS/SFB 10% pH7.0. 50 µL of culture supernatant were added, incubated for 2h at room temperature. After washing, 50 µL/orifice of anti-IL-10, anti-IL-4 and anti-INF-γ biotinylated was added, incubating for 1 hour at room temperature. After further washing, 50 µL of the conjugate avidin-peroxidase was added, incubated for 20 minutes at room temperature and in the dark. The reaction was revealed with an OPD solution in substrate buffer and hydrogen peroxide, reading performed at 450 nm in the Molecular Devices Emax (*precision microplates reader) device*.

STATISTICAL ANALYSIS

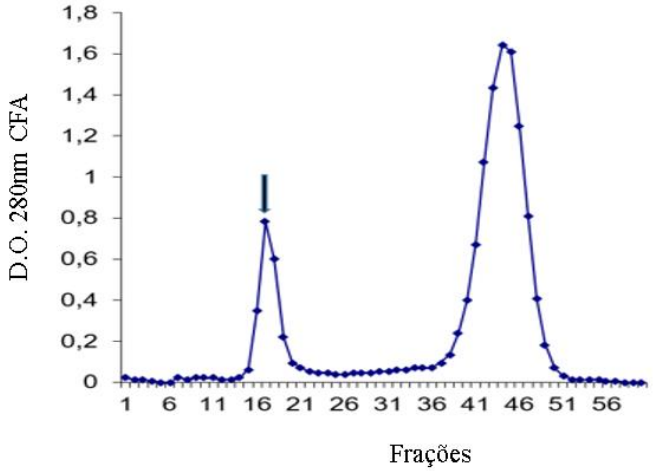
Analysis of variance (ANOVA) and the Tukey-Kramer test were performed. The values described by the mean ± standard deviation, and the results considered significant when $p<0.05$.

RESULTS

CFA CHROMATOGRAPHY (PB18) IN SEPHADEX G-200 COLUMN

The spectrophotometric profile of the CFA Pb18 fractions in Sephadex G-200 Column at 280 nm was shown in Figure 1. The first peak (tube 17) was considered a fraction of hMM, called F17.

Figure 1: Spectrophotometric profile at 280 nm of Pb18 CFA by Sephadex G-200 column chromatography. The arrow (fraction 17) corresponds to the hMM antigen, called F17.

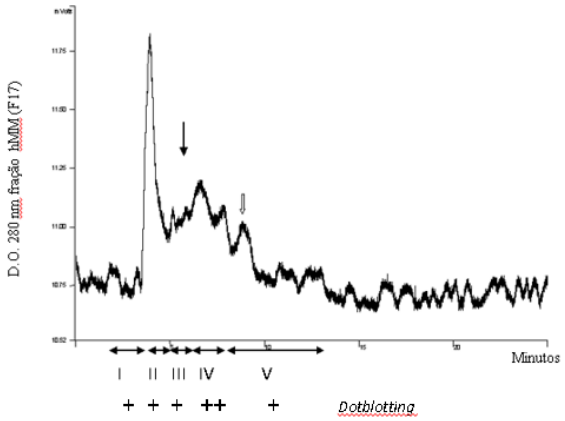


REFRACTION OF FRACTION F17 IN HPLC COLUMN

The refraction of 100 µL of F17 (116.8 µg/ml) in an HPLC column resulted in the spectrophotometric profile at 280 nm shown in Figure 2. The analysis of the fractions eluted

by *dotblotting* resulted in reactive fractions with a pool of sera from patients with PCM. These fractions were mixed forming pools I to V: F17-I (fraction 6 to 9), F17-II (fraction 10 to 15), F17-III (fraction 16 to 19), F17-IV (fraction 20 to 26) and F17-V (fraction 27 to 45), with fraction IV being more strongly reactive. This more reactive fraction was called F17-IV. Considering the MM standards used; egg albumin (45 kDa) and human IgG (150 kDa), the F17-IV subfraction was estimated to have MM between 45-150kDa.

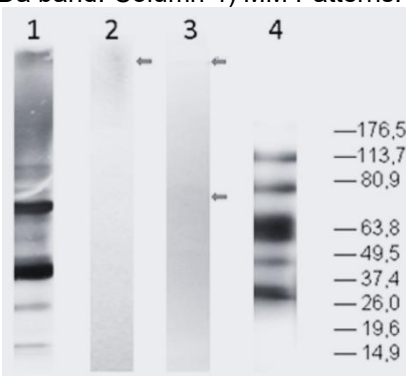
Figure 2: Spectrophotometric profile at 280 nm of the fraction of hMM (F17) refracted in HPLC column C18. Standards: egg albumin 45 kDa and human IgG of 150 kDa. Fractions I, II, III, IV and V were positive by *dotblotting* with a pool of sera from patients with PCM. The hMM sub-fraction in the MM range between 45-150kDa, called F17-IV was the most reactive fraction.



IMMUNOBLOTTING OF HMM FRACTIONS (F1) AND SUB-FRACTIONS (FI-17)

Immunoblotting results demonstrated many bands reactive in CFA with antibodies from PCM patients. In the fraction F17, a band of ~380 KDa was observed (marked with an arrow) and in the sub-fraction F17-IV, a weak band of ~380 KD, a slightly more evident band of ~70 KDa (marked with an arrow) and another weak band around 43kDa, not marked (Figure 3).

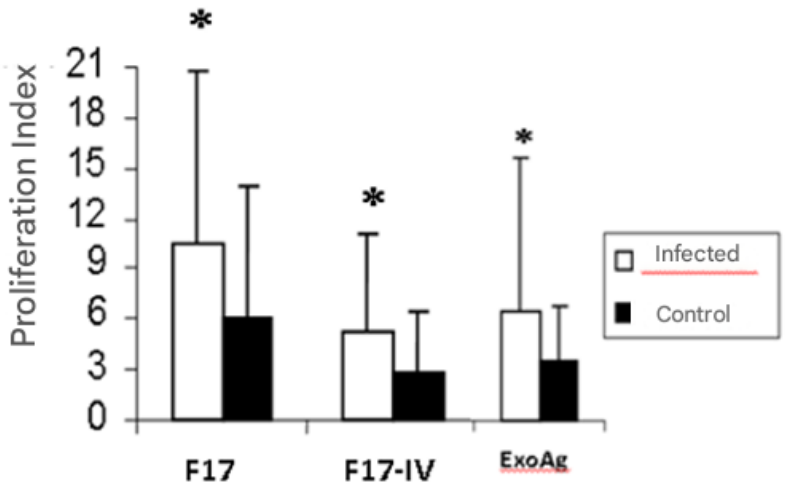
Figure 3: *Immunoblotting results* of the samples: 1) CFA Pb18, 2) F17, 3) F17-IV reactive with a pool of sera from patients with PCM. Arrows in the upper portion of the columns indicate the ~380 kDa band and the arrow in the lower portion indicate the ~70 kDa band. Column 4) MM Patterns.



LYMPHOPROLIFERATIVE RESPONSE ANALYSIS

The lymphoproliferative response to the antigens: F17 (100 µg/ml), F17-IV (50 µg/ml) and Exoantigen (50 µg/ml), expressed as cell proliferation index, was significantly higher in spleen cells from a group of mice infected with Pb18 compared to the uninfected group ($P < 0.05$) (Figure 4).

Figure 4: Cell proliferation index of spleen lymphocytes from Pb18-infected mice and normal mice (as a control) in response to stimuli with the following antigens: 1) F17 (100 µg/ml); 2) F17-IV (50 µg/ml); and 4) ExoAg (Exoantigen) (50 µg/ml). * $p<0.05$ (control versus infected).

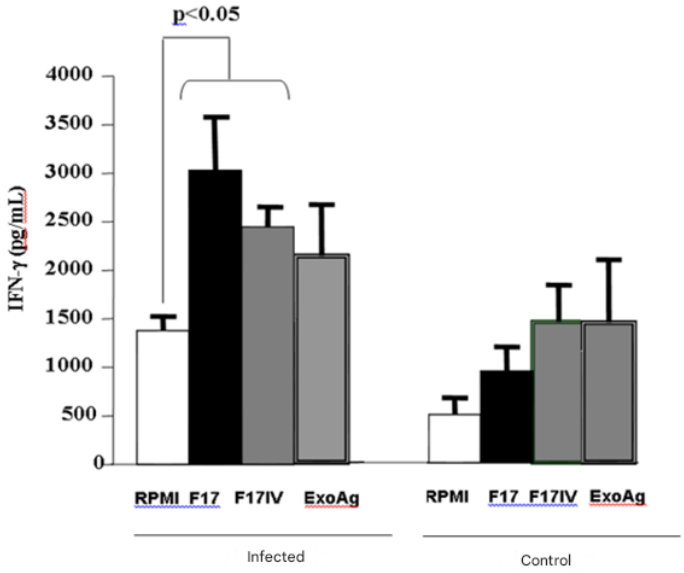


DETERMINATION OF INF-Γ CYTOKINE LEVEL

The results of **INF-γ dosing in spleen cell culture supernatant of** Balb/c **mice** infected with *P. brasiliensis* and stimulated with the ~380 KDa fraction and HPLC product, ~70 kDa subcomponent demonstrated a significant increase **in INF-γ with both components ($p<0.05$) compared to the unstimulated control group, by ELISA.**

However, the stimulation with ExoAg did not induce a significant increase, possibly due to interference of several antigens present in this preparation (Figure 5).

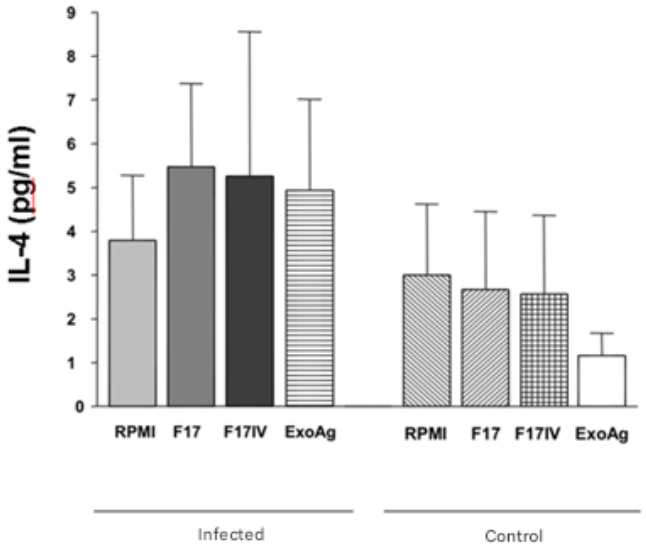
Figure 5: Result of INF-γ level analysis (pg/ml) in spleen cell culture supernatant of Balb/c mice, Pb18-infected group (Infected) and control group without infection (Control), stimulated with F17, F17-IV and ExoAg, by ELISA. RPMI, control without stimulus. RPMI x F17, F17-IV $p<0.05$.



IL-4 CYTOKINE LEVEL DETERMINATION

The analysis of IL-4 levels (pg/ml) in the spleen cell culture supernatant of Balb/c mice infected with Pb18 and the control group, stimulated with the fractions F17, F17-IV and ExoAg, was performed by ELISA. Negative control was represented by the RPMI medium without stimulus. The results showed no significant difference in IL-4 levels between the RPMI control and the groups stimulated with F17, F17-IV and ExoAg ($P > 0.05$).

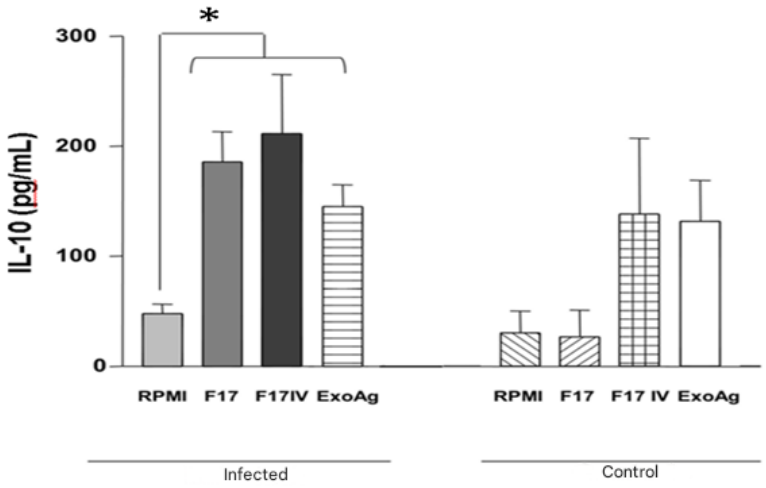
Figure 6: Result of IL-4 level analysis (pg/ml) in spleen cell culture supernatant of Balb/c mice infected with Pb18 (Infected) and control group without infection (Control), stimulated with F17, F17-IV and ExoAg, by ELISA. RPMI, control without stimulus. RPMI x F17, F17-IV, ExoAg p>0.05.



DETERMINATION OF IL-10 CYTOKINE LEVEL

The result of the analysis of IL-10 levels in the spleen cell culture supernatant of animals infected with Pb18 in response to F17, F17-IV and ExoAg showed significant differences in relation to the medium without stimulus ($p<0.05$), Figure 7.

Figure 7: Result of IL-10 level analysis (pg/ml) in spleen cell culture supernatant of Balb/c mice infected with Pb18 (Infected) and control group without infection (Control), stimulated with F17, F17-IV and ExoAg, by ELISA. RPMI, control without stimulus. RPMI x F17, F17-IV, ExoAg p>0.05.



DISCUSSION

Several glycoproteins are excreted/released by *P. brasiliensis* yeast, including gp27, gp43, gp55, gp70, gp87 and high molecular weight polydisperse glycoconjugate (hMM) (Puccia et al., 1986; Blotta & Camargo, 1993; Toledo et al., 1995; Ortiz et al., 1998; Salina et al., 1998). Among these components, gp43 is the most studied because it is released in

greater quantities, is recognized by 100% of the sera of patients with PCM, and is considered a specific antigen. gp70 also stands out (Cezar-dos-Santos et al., 2020), while hMM components are less investigated.

In this work, an antigenic fraction of approximately 380 kDa, called fraction 17, was obtained from gel filtration column chromatography of P. brasiliensis cell-free antigen (CFA) preparation. The approximate molecular mass of 380 kDa was evidenced by immunoblotting. This result is in agreement with previous studies by Marquez et al. (2005), who detected in fraction 17 a component of approximately 366 kDa, within a polydispersion range ranging from 278 kDa to 466 kDa in *P. brasiliensis* B339. Similarly, Pavanelli et al. (2007) obtained a fraction of approximately 380 kDa, also obtained by gel filtration chromatography of CFA of *P. brasiliensis Pb18*.

The presence of this polydisperse hMM glycoconjugate seems to be a common feature in *Paracoccidioides* spp. Fredrich et al. (2010) observed the presence of hMM components in CFAs of *P. brasiliensis* (Pb18) and 20 clinical isolates. The proportion of hMM in relation to the other components ranged from 16% to 20%, indicating a consistent presence of this fraction among the isolates. This fraction of hMM and heterogeneous electrophoretic migration was also detected in the new species *P. lutzii* – LDR2 (Lenhard-Vidal et al., 2018; Assolini et al., 2021), suggesting the constant presence of this component in *Paracoccidioides* spp.

In this study, fraction 17, of approximately 380 kDa, was refractioned by high-performance liquid chromatography (HPLC). The molecular weight of this fraction was initially estimated between 45 kDa and 150 kDa, based on the molecular weight patterns of ovalbumin and IgG, respectively. Subsequently, by *immunoblotting*, the presence of three main bands was verified, corresponding to ~380 kDa, ~70 kDa and ~43 kDa, with the most evident reactivity being ~70 kDa.

These results reinforce the presence of ~380kDa antigen among the polydisperse hMM components in *P. brasiliensis Pb18 antigenic preparations* and associated minor antigenic components that may play an important role in the immune response, especially considering the presence of ~70 kDa bands that are immunoreactive.

The presence of autologous proteolytic enzymes in the CFA preparation was considered, and to avoid their action, the protease inhibitor PMSF was used. However, the PMSF was not able to prevent the generation of lower molecular weight subcomponents. This suggests that, regardless of the presence of enzymes, the hMM component can spontaneously generate smaller components. However, the hypothesis of the existence of

another autologous enzyme resistant to PMSF or the possibility of dissociation of gp70 from the hMM component during chromatographic processes cannot be ruled out.

Puccia et al. (1986) performed the refraction of the first ExoAg peak, obtained by filtration in a column of Bio-Gel P30, in a column of Sepharose-Con A. In this process, they detected the presence of hMM components and glycoproteins smaller than 72 kDa, 55 kDa and 43 kDa. By *immunoblotting*, using serum from patients with PCM, a strong reactivity with gp43 and a weak reactivity with the hMM and gp72 components was observed. These data also suggest that the hMM component generates smaller components. In the present study, HPLC refraction allowed the detection of only ~70 kDa band and ~380 kDa and ~ 43 kDa faintly and ~ 43 kDa. The 55 kDa band was not detected, possibly due to differences in the fungal strains used (B339 versus Pb18), in the types of antigen (ExoAg versus CFA) and in the methodologies applied (Bio-Gel/Sepharose Con A versus Sephadex G-200/HPLC). In addition, the hMM fraction obtained in this study consisted of a single fraction with the highest absorption peak at 280 nm, while Puccia et al. (1986) used part of a broader absorption peak at 280 nm.

The study of the components that induce protection is of great importance, especially for the development of alternative treatment strategies, such as immunotherapeutic procedures, considering the toxicity of the antifungal agents currently used. In this context, the present study evaluated the lymphoproliferative response induced by the ~380 kDa fraction and its ~70 kDa subfraction. Agreeing with the data of Pavanelli et al. (2007), the fraction of 380 kDa was able to induce the lymphoproliferative response in spleen cells from mice experimentally infected with *P. brasiliensis*. Interestingly, the product generated during the purification process by HPLC, a component of MM ~ 70 kDa, retained the ability to induce cell proliferation. This suggests an activation of cellular immunity by both the ~380kDa component and the ~70 kDa component, one of the most relevant defense mechanisms in PCM. The increase in lymphocyte proliferation is an indication that these fungal components have epitopes capable of being recognized by T cells, stimulating the adaptive immune response.

In the evaluation of the cytokine profile in response to the 380 kDa fraction and its subfraction, a significant increase in the levels of INF-γ (interferon-gamma) and IL-10 (interleukin 10) was observed, while IL-4 (interleukin 4) did not present a significant difference. This cytokine profile is characteristic of a Th1-type immune response, which is critical for defense against PCM. INF-γ is a cytokine critical for macrophage activation. Although IL-10 was originally described as a cytokine secreted by Th2 cells, recent studies demonstrate that it can also be produced by Th1, Th2, Th17, Treg cells, dendritic cells, monocytes, and macrophages. IL-10 acts in a dual way on the immune system: on the one

hand, it is a potent anti-inflammatory and immunosuppressive cytokine; on the other hand, it may have immunostimulatory properties (Carlini et al., 2023).

The ~70 kDa subcomponent is possibly a distinct entity from the gp70 previously reported in the literature (Marquez et al., 2005; Biselli et al., 2001; Rigobello et al., 2013). An important evidence of this distinction is its ability to induce an increase in INF-γ, suggesting a differentiated immunomodulatory potential for the Th1 response. This characteristic makes the subcomponent an interesting target for future investigations, as it may represent a new biomarker or a potential immunotherapeutic agent for PCM.

CONCLUSION

The data presented demonstrate the importance of the high molecular weight **(hMM)** components of ***P. brasiliensis***, especially the **380 kDa** fraction and the subcomponents, in triggering the **cellular immune response**. The increase in cytokines associated with the **Th1 response**, such as **INF-γ**, and the absence of an increase in **IL-4**, reinforce the role of these components in inducing an effective immune response in PCM. These findings highlight the relevance of these antigenic components for the development of vaccine or therapeutic strategies for **PCM**.

ACKNOWLEDGMENTS

Araucária Foundation/PR, PROPPG/PROEX, UEL and Coordination for the Improvement of Higher Education Personnel (CAPES).

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