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Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis

Zoilo Pires de Camargo and Marcello Fabiano de Franco

Disciplinas de Biologia Celular e Patologia da Escola Paulista de Medicina, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brasil

Summary

Host-parasite relationship and immunodiagnostic testing in paracoccidioidomycosis have been extensively investigated in recent years. We review the major advances in the understanding of pathogenesis of the disease with emphasis on the sequential steps in granuloma formation and the envolvement of immunological mechanisms in host defenses against the parasite. In addition, the several immunodiagnostic tests used for diagnosis and in the follow-up of patients are commented upon and critically analysed.

Key words *Paracoccidioides brasiliensis*, Paracoccidioidomycosis, Pathogenesis, Fungal immunodiagnosis

Patogenia e inmunodiagnóstico de la paracoccidioidomicosis: Avances recientes

Resumen

En los últimos años, la relación parásito-huésped y los tests inmunodiagnósticos en paracoccidioidomicosis han sido extensamente estudiados. En este trabajo revisamos los importantes avances realizados en la comprensión de la patogenia de la enfermedad con mayor énfasis en los pasos de formación de granuloma y los mecanismos inmunológicos de defensa del huésped contra el parásito. Los diversos tests inmunodiagnósticos usados para el diagnóstico y seguimiento de la enfermedad son críticamente analizados y comentados.

Palabras clave *Paracoccidioides brasiliensis*, Paracoccidioidomicosis, Patogenia, Inmunodiagnosis fúngica

The thermally dimorphic fungus *Paracoccidioides brasiliensis* causes paracoccidioidomycosis (PCM), a human systemic disease, which is confined to Latin America, whose areas of endemicity extend from Argentina to Central America, and constitute one of the most prevalent deep mycosis in this region [1]. On the belief that the disease is acquired when propagules of the mycelial phase of the fungus [2] are inhaled through the respiratory route [3], the infection is thought to take place firstly in the lungs and then may disseminate via the bloodstream and/or lymphatics to all parts of the body [4]. Most infected individuals develop an asymptomatic pulmonary infection; however some patients present with clinical manifestations giving rise to the acute, subacute (juvenile type), and chronic (adult type) clinical forms [5].

Dirección para correspondencia:
Dr. Zoilo Pires de Camargo
Disciplinas de Biologia Celular
Escola Paulista de Medicina
Universidade Federal de São Paulo
04023-062 Rua Botucatu 862/8º
São Paulo, SP, Brasil
Tel.: +55 11 576 45 23; Fax: +55 11 571 5877
E-mail: zoilo@ecb.epm.br

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Pathogenesis

After penetration in the host, the fungus converts into its yeast form, and this process is regarded as a fundamental step for the successful establishment of the infection [4] and the initial phase of the host-parasite relationship. Although the precise interactions between the host and *P. brasiliensis* are not known, the subject has attracted much attention lately.

The fungus

P. brasiliensis isolates vary in their growth curves, ultrastructural features, and antigen and molecular composition. These findings indicate that PCM may be caused by different strains of the fungus.

Very litlle is known about virulence factors in *P. brasiliensis*. Strain virulence influences the host-parasite relationship as demonstrated by the differences in the pattern of granuloma formation and in the organs involved in the experimental infection induced by each strain [6,7]. The prevalence of the clinical forms and the frequency of organ involvement by the disease vary in different endemic areas [4,8]. It is tempting to think that these findings might be related to the presence of different strains of *P. brasiliensis*.

When the infectious propagules reach the host, the first host defenses should be overcome in order for transformation and multiplication into the fungal parasitic yeast form to take place. The first host-parasite interactions occur in the pulmonary alveoli. Here the invasive

capacity of the fungus depends on adaptive mechanisms aimed at resisting the higher body temperature and the phagocyte attack.

There are other mechanisms by which fungal components may influence the virulence of *P. brasiliensis*. Proteinases produced by certain fungi pathogenic in humans have been recognized as potentially important virulence factors. *P. brasiliensis* synthesizes several proteinases capable of hydrolysing casein, collagen, and may have a role in facilitating invasion of host tissues [9].

Attachment or adherence to host cells is a fundamental process by which microorganisms establish infection. The presence of laminin receptor on the surface of yeast-forms of *P. brasiliensis* has been demonstrated. The adherence of fungal cells to a monolayer of epithelial cells was significantly reduced in the presence of antilaminin monoclonal antibodies. The relevance of these *in vitro* data has been tested in the hamster model. Animals infected with *P. brasiliensis* yeast-forms which had been preincubated with laminin, developed a testicular infection significantly more extensive than that of animals infected only with yeast cells [10].

It should be pointed out that, as observed in the ultrastructural study of mucosal lesions from patients, *P. brasiliensis* appears to actively penetrate the mucocutaneous surface and parasitize the epithelial cells, thus evading host defenses and reaching deeper tissues [11]. These data have been confirmed by *in vitro* studies using monolayer cell cultures [12]. The attachment of *P. brasiliensis* to epithelial or alveolar cells may play a crucial role in the process.

The host

Latin American rural workers usually have low socioeconomic levels and present a high degree of malnutrition and chronic alcoholism. In addition, smoking is a very common habit among this population. These preexisting conditions at risk represent important modifying factors of the disease progress since they interfere with granuloma formation and host-defense mechanisms [13].

Recently, the role played by some of these risk factors in the progress of the disease was evaluated in the intraperitoneal model of experimental PCM in rats [14]. The course of *P. brasiliensis* infection was studied in alcoholic animals compared with pair-fed and ad libitum controls. Alcohol per se did not aggravate the infection or affect the specific humoral and delayed hypersensitivity responses. However, the alcoholic rats developed malnutrition which was associated with a severer infection.

Initial host-defense mechanisms

PCM is much more frequent in man, affecting women mainly before the menarcle or after menopause. It has been shown that *P. brasiliensis* has receptors for the 17-β-stradiol in the cytoplasm and that this hormone inhibits the mycelia to yeast transformation [15].

The primary host defense mechanisms interact with the fungus at the portal of entry. If the fungi persist, specific immunological responses are elicited to destroy the parasite. If defense mechanisms are defective, the phagocytes and the immune response do not act efficiently, thus permitting fungal adaptation and transformation.

Some natural defense mechanisms of the host have been investigated in PCM, such a phagocytosis, leukocyte chemotaxis, natural killer (NK) cells, genetic background and complement system.

The role played by phagocytosis in vivo has been demonstrated by the intratesticular inoculation of *P. brasiliensis* in guinea pigs subjected to blockade or stimulation of the reticuloendothelial system and by the intraperitoneal model of murine paracoccidioidomycosis with prior blockade of peritoneal macrophage activation [16]. Extensive exudative-necrotic lesions with large numbers of fungi and a more disseminated type of disease were observed in animals with blockade [17].

In patients, studies of in vitro chemotaxis of circulating leukocytes have shown unimpaired responses suggesting that the phagocytes should have been able to reach the focus of infection. The nature of the chemotactic substances is unknown. However, an intense neutrophilic exudate was obtained when the supernatant of a mixed culture of peritoneal macrophages and *P. brasiliensis* was injected into the peritoneum of normal animals. This soluble factor chemotactic to neutrophils was characterized as a protein of low molecular weight [18].

When, *P. brasiliensis* reachs the pulmonary alveoli, the fungus interacts with alveolar macrophages inducing the release of peptides which attract neutrophils, thus amplifying the response. It has been shown that circulating neutrophils and cells from the bronchoalveolar lavage of patients have normal levels of phagocytic activity, although they fail in their capacity to digest both yeast forms and conidia of *P. brasiliensis* [19-22]. Since these cells were capable of phagocytosing and digesting *Candida albicans*, this may represent an important escape pathway for *P. brasiliensis*

Studies in vitro have shown that murine NK cells limit the growth of *P. brasiliensis*, suggesting that they may play a defensive role during the initial phase of infection. On the other hand, patients' lymphocytes showed a decreased capacity to inhibit *P. brasiliensis* growth in vivro when compared with lymphocytes from healthy controls [23].

Natural defense mechanisms are directly linked to the concept of host resistance or susceptibility to the parasite. Some genetic factors which influence the outcome of the *P. brasiliensis* infection have been elucidated, such as the association between the HLA antigens (A9, B13) and disease, the characterization of resistent and susceptible strains of mice with a dominant pattern of inheritance [24].

P. brasiliensis is able to activate the complement system [25], resulting in an oposonizing effect which facilitates fungal phagocytosis by macrophages.

Immunological mechanisms

When the fungus invades tissues, the interaction of its antigens with the host's immune system causes an immunological response which exerts a fundamental influence on the outcome of infection. Even though the two arms of the immune system – humoral and cellular – have specific and independent functions, it is their interaction that guarantees host survival.

Patients with PCM do not show a deficiency of antibody production but rather a hyperactivity of the humoral immune response, which results in high antibody titers and hypergammaglobulinemia. The anti-*P. brasiliensis* antibodies may play a role in host's defenses against the parasite by enhancing opsonisation of the parasitic fungal cells and therefore the phagocytic activity of phagocytes. In addition, antibodies interact with interstitial and circulating antigens, resulting in immune complexes, which may downregulate the specific cellular immune response.

As in other deep mycoses, cellular immunity constitutes in an crucial host defense in PCM. There are several evidences that *P. brasiliensis* evades host defenses by

inhibiting the cellular immune response. The mechanisms by which the fungus induces this immune depression are not fully understood. This include several factors that may interact in a multifactorial way, such as plasmatic factors, circulating antigens, immune complexes, specific antibodies, specific suppressor T-cells and extensive granulomatous involvement of lymph nodes.

The paracoccidioidal granuloma

PCM is a granulomatous disease which is characterized by the formation of epithelial tubercles with central areas of necrosis, aggregates of polymorphonuclear leukocytes, a lymphomononuclear halo and fibrosis. On the whole the tissular host response to the presence of the parasitic fungal cells is a typical epithelioid granulomatous inflammation with variable numbers of parasitic cells and areas of suppuration.

The granulomatous inflammatory response with formation of epithelioid tubercles is assumed to be the most evolved form of tissue reactivity and the most effective biological defense weapon against invading microorganisms [26].

Both direct and indirect evidences seem to indicate that *P. brasiliensis* granuloma is intimately related to the immune response of the host. Thus, the paracoccidioidal granuloma may represent an immune-specific tissue response of the host to the fungus in an attempt to destroy, block, and circumscribe the parasite, and to prevent its multiplication.

Direct evidence that the paracoccidioidal granuloma is related to the host-cell-mediated immune response is provided by data on human and experimental animal infection. Fava Netto [27] was the first to point out this fact by classifying the clinical forms of the mycosis into two types: (1) a benign, localized infection with persistent cellular immune responses and histopathology showing compact epithelioid granuloma with few fungi and (2) a disseminated infection with a mixed suppurative and looser granulomatous inflammation, showing extensive areas of necrosis and large numbers of fungal cells. More recently, these two forms were redefined as the hyperergic and anergic poles of the disease, respectively [28].

The same pattern of correlation has been documented in experimental infections of normal and athymic (nu/nu) mice. In contrast to animals with an intact T-cell system, the nu/nu animals showed high mortality with lesions containing few granulomas but numerous fungi. Further experiments have demonstrated that even when athymic animals succeed in organizing a granulomatous inflammation, the absence of cellular immune responses prevents killing of the fungi [29]. These data were interpreted as evidence for the absence of lymphokine stimulation of the macrophages fungicidal activity. Such observations are similar to those reported in PCM patients with AIDS or in those receiving immunosuppressive therapy [30].

Microanatomic studies of the *P. brasiliensis* granuloma by histochemistry and immunohistochemistry have demonstrated that T cells form a peripheral mantle around the centrally aggregated macrophages. The majority of the lymphocytes are T-helper cells with few suppressor cells, indicating that those cells are actively involved in the pathogenesis of the lesions and in disease control [31,32].

The histiocytic population has been studied using as markers the enzyme lysosyme (lys) and the nerve tissue specific antigen S-100 protein. The phagocytic histiocyte is lys+ S-100- whereas the antigen presenting histiocyte is lys- S-100+. In the granulomata, the histiocyte population was predominantly lys+ with few peripheral S-100+ cells, a pattern similar to that of other immunogenic granulomata. The interplay at the peripheral halo between antigen presenting cells and lymphocytes would result in the release of T-cell stimulating factors, such as interleukin 2. Lymphokines released by activated lymphocytes would then attract, fix and activate macrophages in the inflammatory foci. The activated macrophages would show enhanced killing of *P. brasiliensis*, secrete cytokines and further differentiate into epithelioid cells and giant cells.

The neutrophils tend to concentrate around the fungal cells and are stimulated in their killing activity by locally released cytokines.

In addition, *P. brasiliensis* granuloma show a variable number of NK cells and eosinophils, whose major basic protein involves fungal cells and may participate in the killing of the parasite [33].

Finally around the tubercles there are numerous B lymphocytes and plasma cells, which are mainly IgG producing cells. The locally produced specific anti-*P. brasiliensis* antibodies diffuse into the granulomata and immunoprecipitate the antigens, facilitating the elimination and preventing the diffusion of fungal components.

Altogether the major effector cells in the paracoccidioidal granuloma are T lymphocytes, of the helper and cytotoxic subsets, activated macrophages, including epithelioid and giant cells, activated polymorphonuclear leukocytes, both neutrophils and eosinophils, NK cells, B lymphocytes and plasma cells.

More recently, several in vivo and in vitro data have highlighted the role played by Th1 and Th2 cytokines in host defense in PCM: i) Th1 cytokines (γ-interferon, IL-2, IL-12) are associated with host resistance against infection; ii) Th2 cytokines (IL-4, IL-5, IL-10, TGF-α) are associated with host susceptibility to infection; iii) the *in situ* detection of cytokines in murine models of PCM have revealed an association between presence of IFN-γ and resistance, and IL-4 and susceptibility to infection [34-37].

However, some additional studies have not shown a Th1/Th2 polarization in regressive and progressive forms of human and experimental PCM.

The significance of these cellular regulations and interactions and the role played by cellular immunity in the host defenses against *P. brasiliensis*, at the tissular level, are underlined by the great amount of in vitro studies done in PCM patients, such as reduced T-cell expression of IL-2 receptors in peripheral blood [38], and enhanced pulmonary macrophage fungicidal activity induced by lymphokynes [39], enhanced peripheral neutrophil fungicidal capacity in sensitized mice [40].

In accordance with these data, studies on protective immunization in PCM have indicated a positive correlation between elevated cellular immune responses and restriction of infection and in the numbers of fungal cells in granulomata [41,42].

T-lymphocyte immunostimulants (e.g. levamisole, IFN-γ) have been used in experimental models of disseminated PCM. The results indicate restoration and partial maintenance of the cellular immune response, with a more benign course [43]. The same effect has been obtained with transfer factor from normal or specifically sensitized animals [44].

In humans, glucans from *Saccharomyces cerevisiae* have been tested in conjunction with antifungal drugs in the treatment of severe forms of PCM. The use of this non-specific immunostimulant resulted in marked response to treatment, with rapid full recovery and decrease in the number of relapses [45].

These pioneering studies on immunotherapy have opened new avenues for treatment based on the use of the host defense system itself. Studies of the immunoregulatory mechanisms and the interactions between lymphocytes and macrophages in PCM are recent. These are rapidly developing areas of research which will make important contributions to the understandig of the pathogenesis of the mycosis.

Immunodiagnosis

In the management of a suspected PCM infection, no diagnostic test is superior to the isolation of the causative agent from a relevant clinical specimen, or to its unequivocal physical identification in clinical specimens and/or in histopathological setting of tissue invasion. However, these ideal situations are not always possible, and so one must often employ diagnostic approaches that are based on serologic testing. In the case of PCM, serologic testing, such as immunodiffusion, is so reliable that a positive test, even if the titer is low, is indicative of infection.

The last decades show a rapidly expanding field of immunology permitting the development of several new techniques, which were gradually being adopted by researchers interested in the diagnosis of fungal diseases, and several methods were currently being used as possible aids in diagnosing some mycotic infections, mainly PCM. Much of those serologic assays utilize polyclonal/monoclonal antibodies or antigens which can be easily quantified with high sensitivity and have resulted in methods with more sensitivity and specificity.

The successful application of serological tests for diagnosing mycotic infections is very important because the incidence and mortality of the mycoses, particularly those caused by opportunistic fungi has shown a marked increase in recent years, especially in patients whose immune defense mechanisms have been compromised by severe underlying diseases such as AIDS, cancer, and hematologic disorders. This emphasizes the utility of standardized techniques and antigens as diagnostic tools in the clinical and laboratory evaluation of patients with suspected fungal infections. Fungal antigens are of great interest not only for the study of their fundamental properties, but also because of their use as reagents for the diagnosis of fungal diseases.

The scientific literature on the immunology of the mycoses for the last years shows an increasing number of papers dealing with serological testing and different antigenic preparations. In the early 1980s, Restrepo [46] reviewed the extensive literature about the serodiagnosis of PCM, and in the early 1990s, Mendes-Giannini *et al*. [47] wrote an excelent chapter about the same subject. However, in the early 1970s, Fava Netto [48] and Negroni [49] had also documented the problems about antigens for PCM diagnosis.

As there is extensive and diverse literature about serology in PCM, in this article we will mainly discuss our laboratorial experience, and the most important advances in the PCM serology, in order to show the current knowledge about the immunodiagnosis of PCM.

Antigens. For many years, various different antigenic preparations have been used for the serodiagnosis of PCM. However, these various antigens lacked a standardized preparation from one laboratory to another and included sonic extracts from the yeast forms, concentrated filtrates, and lyophilized filtrates to cite just a few approaches. Moreover, each antigen was prepared from cells grown in different culture media and under different growth conditions, such as incubation time, growth temperature, size of initial inoculum, and shaken or stationary cultures. It is possible for different antigenic preparations to vary considerably in activity and quality as a result of lack of proper lot-to-lot production standards even within the same laboratory. Different strains of *P. brasiliensis*, on the other hand, can produce remarkably different qualities and quantities of antigens, a response that is also influenced by the growth medium. With the variety of problems in producing a proper *P. brasiliensis* antigen, it is not surprising that there is considerable disagreement regarding the sensitivity of the tests for the serodiagnosis of PCM [50-56].

The most important contribution in the serology area was the identification and purification of the 43 kDa glycoprotein (gp43), and initially designated as an specific diagnostic antigen [57]. This glycoprotein is identical to the E2 antigen described early by Yarzabal *et al*. [58], and is indeed the molecule responsible for the specific immunoprecipitation in ID test (band 1) described by Restrepo and Moncada [59] and the arch A obtained in immunoelectrophoresis by Restrepo and Drouhet in 1970 [54]. The gp43 is the main exocellular antigen of *P. brasiliensis* yeast phase and its synthesis at the ribosome level is done by active transport to the cytoplasmic membrane, where is excreted by simple exocytosis or through the lomasomes [60]. Gp43 can be easily purified by affinity column.

The specificity of the serologic tests depends overall on the antigen used. *P. brasiliensis* crude antigens have components that are common with other fungi, and in this way, sera from patients with other mycotic infections may cross-react with it, mainly histoplasmosis and Jorge Lobo's disease. For this reason, in the last years, great efforts have been made in order to obtain good antigenic preparations which can be used in serological tests with specificity and high sensitivity. Camargo *et al*. [61] standardized the process to produce an antigenic preparation useful for PCM serodiagnosis. The exoantigen obtained after seven days of growth showed to be ideal for immunodiffusion tests and was named Ag7. This antigenic preparation was obtained with the strain B-339, well known since the early 1960's, for producing stable crude antigens. *P. brasiliensis* culture's of this preparation was standardized by the number of yeast cells in the initial inoculum (approximately 8×10^6 cells) and a final lyophilized product of a pool of 6 batchs. The choice of seven days-old-cultures was because the growth cells' were in the middle of the exponential phase, before the decrease of the number of viable cells, when autolysis starts with liberation of proteases from the cytoplasm of dead cells, that also prove a problem. Under this protocol, the main component of the exoantigen is gp43, the immunodominant antigen. Over the years, the methodology was modified so as to simplify the process of production of the Ag7, accessible to all laboratories, even those with less resources. Nowadays, we prepared the Ag7, by inoculating the slants of 5 to 10 yeast cultures tubes in 500 ml of medium, and culturing it during seven days, on a giratory shaker at 35°C, when the filtrate is obtained, concentrated 20-30 times, dialysed, and protein content determined. In this moment the Ag7 is tested against a battery of previously confirmed PCM serum. If all selected sera show positive reactions, this antigen is aliquoted and conserved at –20°C until use. However, if some control sera do not react, the antigen is further concentrated and tested again. In general, the protein content is about 300 to 500 μg per ml, by the Bradford method [62]. In our experience, gp43 is abundantly secreted after seven days in different broth

media. In almost all preparations, when the exoantigen is analysed by SDS-PAGE, the gp43 represents 80-90% of the preparation.

Immunodiffusion. During the last years immunodiffusion (ID) has been the test of choice for the initial diagnosis of suspected patients of PCM. The ID test has high specificity and sensitivity may vary from 65 to 100% depending on the kind of antigen used [47,63]. Recently, the Ag7 was tested in ID by a multinational group of researchers , with the aim of providing regional laboratories, within the endemic areas, with an accurate yet simple test to promptly diagnose PCM [64]. The results showed that ID test was 84.3% sensitive and 98.9% specific, concluding that in those conditions the Ag7 can be regarded as an important diagnostic tool in PCM. However, in our laboratory false negative results have been observed in the order of 2-3%; these observations have also been noticed by others [65]. In these cases, the PCM patients have severe disease mainly with important pulmonary involvement; on the other hand, the diagnosis is easily given by the finding of budding yeast cells in the sputum where *P. brasiliensis* is abundant and easily found by direct examination with 30% KOH. It is our oppinion that in these situations and in the moment of diagnosis, the immune system of the patient is depressed, and there is no sufficient antibodies to precipitate in ID test. The paracoccidioidin skin test is also negative. So the patient receives the initial treatment and after a period of one to two months, immunity is restaured and ID test becomes positive (personal observations). Itano [66] has proposed that this problem may be due the presence of IgG asymmetric antibodies which possess a structure resulting from a predominant mannose-rich oligosaccharide moiety linked to the Fd part of only one of the Fab arms of the antibody molecule. As a consequence they are functionally univalent, non precipitating.

Counter-immunoelectrophoresis (CIE). CIE is another test used to provide early diagnosis of PCM. Like in ID test, the antigens used varied from laboratory to laboratory, and they are extracts from sonicated yeast cell suspensions, exocellular antigens from yeast or mycelial cultures, cell free antigens obtained from yeast cells, and cross-reactions are due to the use of unfractionated antigens. CIE is regarded as having a sensitivity equal or slightly greater than that provided by ID [67]. Some authors related sensitivity between 77 and 97% and specificity at least 95%. Some laboratories suggest CIE as the first routine screening of sera from suspected PCM patients, because the speed in which results can be obtained. In our experience, the time results in CIE is pratically the same as in ID test. After electrophoresis, the glass slide need to be washed for 24 h, dried for further 24 h, and stained. This is the same time necessary for ID test. On the other hand, in CIE, it is necessary to use buffered agarose, buffer and a power supply. These conditions are not always accessible for laboratories in endemic regions of PCM, in underdeveloped countries [for more details about CIE see ref. 47].

Solid-phase immunoassays. Solid-phase immunoassays, especially enzyme immunoassays (ELISA), are popular for the quantitation of antibodies both in research and clinical medicine. Enzyme immunoassays employ antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic moiety is preserved. These assays give reliable and reproducible results and are extremely sensitive. The basic principle of the method is the use of detector antibody reagents bound to an enzyme indicator. The detector antibody is chosen to react specifically with an antigen or antibody component of interest. The amount of detector bound to an antigen or antibody can then be estimated by quantifying the enzyme activity that remains associated with the system after the unbound reagent is washed off. Because a single enzyme molecule can catalyse the conversion of millions of substrate molecules, the "signal" of bound detector molecules is therefore greatly amplified, giving the ELISA system a very high sensitivity. ELISA is relatively simple to perform, gives quantitative results, is highly sensitive and the reagents are long lived, can be safely shipped and conveniently stored. Furthermore, different classes of immunoglobulins can be determined thus allowing a more precise evaluation of the humoral immune response.

Pons *et al*. [68] were the first to introduce ELISA for detection of anti-*P. brasiliensis* antibodies. ELISA has since been the basis of many other publications for detection of serum antibodies to *P. brasiliensis* [69,70-72], and has been used with a range of different antigens, since crude mixtures, partially purified and purified molecule as gp43, and generally have high sensitivity, but not necessarily been coupled with high specificity. Mendes-Giannini *et al*. [69] and Camargo *et al*. [70] give a good account to the development of the methodology of ELISA for anti-*P. brasiliensis* antibodies. Mendes-Giannini *et al*. [69] standardized ELISA using a *P. brasiliensis* yeast filtrate as an antigen, and found 100% sensitivity and 88% specificity, considering a cut off of 1:40. However, cross-reactions were observed with sera from patients with histoplasmosis and Jorge Lobo's disease. To overcome this cross-reactivity, the authors standardized an ELISA-abs test, in which the serum sample is first absorbed with *Histoplasma capsulatum* yeast cells and also with a mycelial filtrate. Camargo *et al*. [70] when evaluating the serological response of PCM patients' sera by ELISA, and using *P. brasiliensis* yeast filtrate verified that the previous absorption of the sera with dead cells of *Candida albicans* was necessary to eliminate the cross-reactivity with heterologous sera. The level of sensitivity and specificity achieved with ELISA was 95% and 93.4%, respectively, considering a cut off titer of 1:400. These filtrates are constituted mainly by the glycoprotein gp43, the immunodominant antigen. This molecule has also been tested in ELISA, since when it was known as the E_2 fraction [56]. Mendes-Giannini [71] verified that E_2 (gp43) antigen did not cross-react, in ELISA, with histoplasmosis, aspergillosis, Jorge Lobo's disease, sporotrichosis, tuberculosis, leishmaniasis sera when previously adsorbed with a filtrate of *H. capsulatum*. Puccia and Travassos [72] also tested gp43 in ELISA and immunoprecipitation with ^{125}I-labelled antigen, and verified that most of the PCM sera recognized primarily peptide epitopes of the gp43; however some PCM sera were able to recognize carbohydrate epitopes and accounted for up to 45% of the total reactivity. 53% of the histoplasmosis sera reacted within the same range of the PCM sera. More recently, Camargo *et al*. [73], testing gp43 in antibody capture ELISA, were able to discriminate among PCM and heterologous sera (histoplasmosis, Jorge Lobo's disease, candidiasis, aspergillosis). In this study, the mininal titer obtained with PCM sera was 1:51,200 and the maximal titer was 1:819,200; the arithmetical mean titer was 1:549,093. Only one serum from Jorge Lobo's disease and one serum from aspergillosis had maximal titer of 1:51,200. The sensitivity of the capture ELISA was 100% and the specificity was 96.7%, considering the end point of reactivity. However, if the analysis is made according

to the O.D., e.g., at the 1:800, 1:1,600, 1:3,200, etc., serum dilutions, there occur enormous differences among homologous and heterologous sera. At these dilutions, the capture ELISA is 100% specific. In the same study [73], gp43 was also analysed by conventional ELISA, with PCM and heterologous sera, showing that heterologous sera reacted in the range of PCM sera, mainly histoplasmosis and Jorge Lobo's disease sera. These contradictory results can be explained by how gp43 is presented to be recognized by antibodies in different tests. When gp43 is fixed onto solid substrates, as in ELISA plates, made of plastic, there are conformational changes in the molecule, that cause the exposition of carbohydrate epitopes recognized by heterologous sera; when the molecule is in solution, as in capture ELISA (gp43 is fixed on MAb anti-gp43), gp43 assumes different conformation, presenting epitopes more reactive with specific antibodies, those directed against protein epitopes. Deglycosylation of gp43 frequently abolishes the reaction of the heterologous sera with this antigen [74]. The antibody capture ELISA represents an advance on the detection of specific antibodies, once it uses specific monoclonal antibody against gp43, which is fixed on the plates; however the users depend on this MAb. Classical ELISA is really an excellent format for the detection of antibody response in PCM for many laboratories. As seen in various reports, gp43 is not a totally specific molecule, since its carbohydrate composition contains epitopes recognized by heterologous sera, mainly by histoplasmosis. However, one can perfectly separate the heterologous sera, mainly histoplasmosis sera, by the OD diferences in each serum dilution. Gp43 may be considered a specific diagnostic antigen for PCM, provided the presentation in classical ELISA when the molecule is fixed directly on the plastic, or in capture ELISA when it is fixed on the Mab anti-gp43, and also how we interpret the results, considering the end point of reactivity or the OD in each serum dilution.

Immunoblotting. Immunoblotting is a procedure in which antigens separated by polyacrylamide gel electrophoresis are transferred (blotted) to nitrocellulose sheets, allowed to react with antisera, then sites where antibodies have bound to antigens are revealed by means of enzyme detector reagent and specific substrate. The procedure has been used to identify *P. brasiliensis* antigens that are recognized by antibodies in patient's sera [75-78].

Camargo *et al*. [75], using a *P. brasiliensis* yeast exoantigen, standardized the Western blot for diagnosis and follow up of PCM patients. Anti- *P. brasiliensis* IgG reacted with four major components of 70, 52, 43 and 20-21 kDa. The 43 kDa glycoprotein was the predominant IgG reactive antigen, recognized by 100% of the patient's sera; the 70 kDa antigen was recognized by 96% of the PCM sera. Cross-reactivity could be minimized by serum dilution. In this study the authors considered gp43 and gp70 as marker for human PCM. The authors studied 25 sera from patients prior antimycotic therapy and 72 sera of patients under therapy; the later patients presented an decrease of antibody titers against gp43 and gp70. In other study, Blotta and Camargo [78] studying 60 PCM patients' sera (30 acute form and 30 chronic form) found that gp43 was recognized by 100% of sera (IgG), corroborating previous findings. Mendes-Giannini *et al*. [77] studied the Western blotting assay based the profile showed by sera from patients with the distinct forms of PCM, both before and after therapy. The majority of the sera recognized gp43, but other molecules were also recognized. Although Western blotting is highly sensitive and an clear assay, it is not accessible for all laboratories in the PCM endemic regions of Latin America. However, Taborda and Camargo [79], using an other format, a dot immunoassay, could provide a more user-friendly means of antibody detection for PCM diagnosis. In this circumstance, they treated gp43 with sodium metaperiodate to abolish cross-reactivity with histoplasmosis sera, obtaining specific reactions. The amount of gp43 coated in the nitrocellulose membrane was 400 ng/dot.

Patients' follow up

There is no consensus among the physicians about the follow up of PCM patients under antimycotic therapy. Each group adopts his own procedure according to its own experience, so that different tests are adopted.

Mendes-Giannini *et al*. [47] showed by Western blotting a significant fall in the reactivity of the anti-gp43 IgG during recovery, while increased reactivity occurred during relapses. Camargo *et al*. [75], also using Western blotting, showed that antibodies anti-gp43 and 70 decreased significantly in patients undergoing antimycotic therapy, and both molecules can be considered to be markers for human PCM. Mendes-Giannini *et al*. [71] have demonstrated that the decrease of anti-gp43 IgG, IgA, and IgM, measured by ELISA, correlated well with clinical improvement.

In a serie of studies, Campos *et al*. [80-82] stressed that it is difficult to correlate CF and indirect immunofluorescence (IIF) tests with clinical remission criteria in long-term evaluation of PCM patients. Campos *et al*. [83] analysed and compared CF, CIE, ID, and Magnetic ELISA (MELISA) tests in the follow up of PCM patients. Significative correlation was obtained between CF and CIE tests with clinical maintenance or relapse of PCM patients. MELISA and ID tests were able to distinguish remission of maintenance or relapse in these patients. This fact was not observed with CF and CIE. The anticomplementary sera in CF tests were identified by ID and MELISA tests. So, in this study, ID and MELISA represent an advantageous alternative to CF in diagnosis and during follow up of PCM patients. Mendes [84] restudied the serological test of ID, CIE, CF, and IIF, before treatment and during the follow up of PCM patients. The comparison of the serological tests, according to the sequence of dilutions and considering significant the differences of more than one dilution, revealed that the agreement between tests ranged from 54.0 to 80.3%. The correlation between ranges of serological levels at different tests showed agreement between 87.6% and 98.8%. The time necessary for regression to negative results was 15 to 19 months for ID, and 23 months for IIF (there is no data for CF). The authors enphasized that the differences observed in the time necessary for regression to negative results, the low sensitivity of ID test and the low specificity of IIF, confirm that the treatment must be maintained for one year after achievement of a negative serology.

Alves [85] analysed and compared three differents methods for the follow up of PCM patients, ID, classical ELISA, and capture ELISA (IgG). The sera were collected before treatment, and 6, 12, and 18 months of therapy. The study concluded that ID and capture ELISA titers had a significant fall after six months in those patients with good clinical evolution, while in classical ELISA, the titer fall occurred only after 12 months of treatment. However, no test was able to detect relapse in the PCM patients studied. The author concluded that ID is the better option among the 3 tests studied for the follow up of PCM patients.

Final remarks

At the moment great advances have been reported on the immunopathology of the mycosis. The standardization of experimental models, utilizing genetically controlled susceptible and resistant mice, have clarified many aspects of the host-parasite in the mycosis. Moreover, the identification of the gp43 gene and the subsequent synthesis of the molecule have encouraged experiments on the vaccination of experimental animals against an infectious challenge; the pilot results are promising. The better understanding of the molecular biology of the parasite have opened avenues for the use of modern techniques in the identification of the parasiste in clinical samples, such as *in situ* hybridization in biopsies, as well as in the molecular analysis of strains isolated from hosts or soil. Finally the immunopathogenesis of the fungal granulomatous inflammation has been deeply investigated using monoclonal antibodies for the identification of the inflammatory cellular components and their locally secreted cytokines, as well as using experimental animals depleted or knockouted of cells and receptors for mediators.

Diagnosis based on antibody detection is useful for the diagnosis of systemic mycoses – mainly PCM and HPM – and its sensitivity and specificity depends both of the antigen and test used. Unfortunately, there are antibody tests that employ crude antigens suffering from poor specificity and sensitivity. Consequently, there occur variable degrees of cross-reactivity among the serology of mycotic diseases. Thus, this limits the value of antibody tests based on crude antigen mixtures. Nowadays, the principal molecules of various fungi are already known and can be purified and tested for immunodiagnosis. For example, we can cite the 43 kDa antigen of *P. brasiliensis*, the 94 and 120 kDa molecules of H. capsulatum that are being used and tested in their native and chemically treated forms, with very promissing results. On the other hand recombinant molecules are also being assayed in various serologic tests. The 140 kDa recombinant protein of H. capsulatum and the 27 kDa recombinant molecule of *P.brasiliensis* [86] are good and promising examples.

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