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Interactions of *Paracoccidioides brasiliensis* with host cells: recent advances

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Abstract Host-fungal interactions are inherently complex and dynamic. In order to identify new microbial targets and develop more effective antifungal therapies, it is important to understand the cellular and molecular mechanisms of disease. Paracoccidioidomycosis provokes a variety of clinical symptoms, and *Paracoccidioides brasiliensis* can reach many tissues, but primarily attacks the lungs. The ability of the pathogen to interact with the host surface structures is essential to further colonization, invasion, and growth. Epithelial cells may represent the first host barrier or the preferential site of entry of the fungus. For this reason, interactions between *P. brasiliensis* and Vero/A549 epithelial cells were evaluated, with an emphasis on the adherence, induction of cytoskeletal alterations, and differential signaling activity of the various surface molecules. The adhesion to and invasion of epithelial cells by *P. brasiliensis* may represent strategies employed to thwart the initial host immune response, and may help in the subsequent dissemination of the pathogen throughout the body.

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Introduction

Paracoccidioidomycosis has a multiplicity of clinical presentations, from cutaneous to systemic forms, and can attack various tissues, especially in the lung [1]. *Paracoccidioides brasiliensis* and other fungi that cause systemic mycoses use a sequence of different mechanisms to become established in the host, from the first contact with host cells until the later stages of the disease. These mechanisms need to be better understood. In particular, those involved in dissemination are not at all clear; neither are the steps, by which the fungus crosses to the intravascular compartments of various organs [2, 3]. The different clinical forms of the disease and the occurrence of asymptomatic infection may be a result of host-related factors, such as sex, age, and immunological status, as well as characteristics of the infecting agent, especially its virulence [4]. Fungi are non-motile eukaryotes that depend on their adhesive properties for selective interaction with the host cells [5].

Host-fungal interactions are inherently complex and dynamic. In order to identify new microbial targets and develop more effective antifungal therapies, it is important to understand the cellular and molecular mechanisms of the disease [6]. Recently, the

implications of evolution for host-parasite interactions have led authors to investigate how heterogeneities in parasite virulence and host life-history may affect the persistence and spread of diseases in natural systems [7]. On the other hand, new virulence factors are being identified in many infectious microbes, and some research focuses on the relative contributions of virulence factors to pathogenesis [7, 8]. The characterization of some of these factors has demonstrated that virulence is complex and multifactorial [9]. Many fungal pathogens, such as *P. brasiliensis*, have multiple virulence factors that can damage host defenses, and thus, contribute to an overall virulence phenotype for that organism. At the moment, the attachment, colonization, and dissemination of the etiological agent are considered to be the crucial stages in the onset of disseminated mycosis [10, 11].

Many fungal genes have been described as probably involved in the survival of *P. brasiliensis* in the host. Among these genes are those that encode the proteins essential to the life of the fungus, and those encoding proteins indispensable to the interaction with the host, supplying a pathogenic phenotype [12–16]. However, up to now, none of these genes has been confirmed as important in fungal virulence. More efficient molecular genetic systems for transformation and gene expression have been described only recently, and these will provide new opportunities to study the role of *P. brasiliensis* genes in pathogenesis [17]. It is likely that this fungus will produce a plethora of virulence factors.

The adhesion of the infectious propagules of *P. brasiliensis* during infection of host cells is a crucial first step to subsequent invasion, colonization, and growth, in which various phenotypes (growth, invasion, and metastasis) are developed, depending on the fungal strain, the host, and other factors [10, 18–21]. Secondly, the internalization of yeast cells of *P. brasiliensis* by epithelial and endothelial cells has also been demonstrated [22, 23]. This could be a mechanism, by which the microorganisms gain access to the bloodstream and spread to other tissues.

Interactions of epithelial and endothelial cells with *P. brasiliensis*

The airway epithelium represents the primary site for contact between airborne microbes and their hosts. During human infection with *P. brasiliensis*, the first cells to encounter the organisms may be alveolar macrophages and alveolar epithelial cells. Although epithelial cells serve as a relatively passive physical barrier to infection, they may contribute more actively to signaling events in the immune response. To cross tissue planes and cause invasive disease, the fungus must invade normally non-phagocytic host cells of the epithelium and endothelium [11]. The presence of typical yeasts of *P. brasiliensis* on and inside epithelial cells was first demonstrated 40 years ago [24]. This feature was observed in neutrophils and in the chorioallantoic membrane, as well as in mucocutaneous surfaces of infected human tissues. Structures were observed with a surrounding vacuole membrane and no cell wall, similar to the spheroplasts of *P. brasiliensis* [25]. Techniques that employ mammalian cell cultures can be used initially to give insights into host-parasite interaction. A cell line derived from human alveolar epithelial cells, A-549, has been used as an in vitro type II pulmonary epithelial cell model, as have Vero and HeLa cells [26, 27]. These models have been developed to study the steps that occur between the initial contacts of *P. brasiliensis*, and the events that culminate in its entering the cell [28, 29]. The adherence phenomenon varies among strains and correlates with their virulence [30]; strains that are more virulent in animals exhibit enhanced adhesion in vitro. In particular, Pb18, described as the most virulent strain in animals, adhered most strongly to Vero cells [28].

One isolate of *P. brasiliensis* (Pb113) was seen to adhere to epithelial cells [31], and then apparently invade them. Initially, the yeast forms adhered to the epithelial cells and, some time later, were observed in the cytoplasm, close to the nucleus [22, 29]. The adhesion to the epithelial cells was accomplished by means of a small tube [26, 27], and alterations in the Vero cell membrane were then observed around the adhesion area, with fungal elements, forming depressions probably caused by extracellular products of the yeast, perhaps proteinases. Characteristic fungal and protoplast forms were observed inside the cells after 24 h of incubation (Figs. 1, 2). Some fungus has been observed enclosed in a vacuole membrane, suggesting phagocytosis [28]. The fungus probably enters the cell as a protoplast, and regenerates its cell wall in the host cytoplasm. This event requires further study. In HeLa cells, a similar phagocytosis was seen after

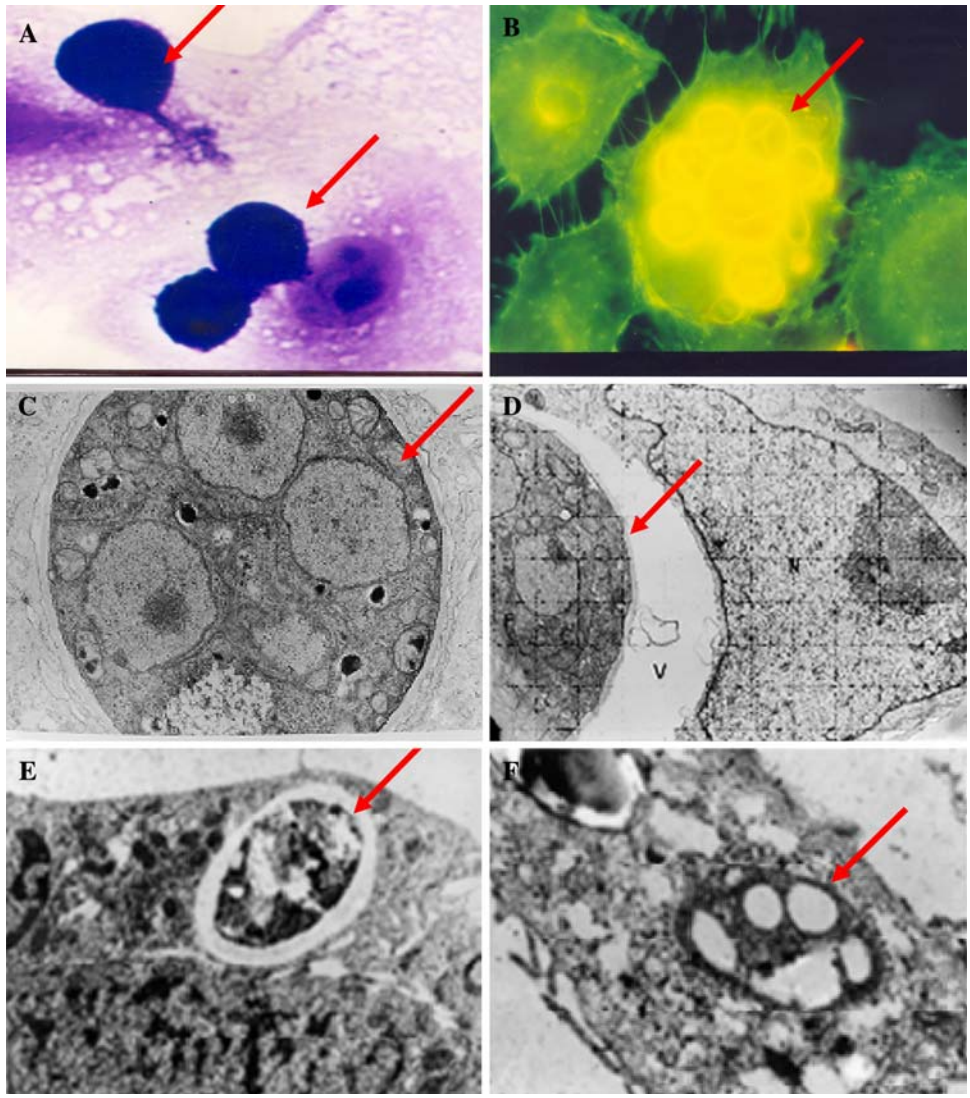

Fig. 1 Adhesion to and invasion of Vero cells by *P. brasiliensis*. (**a**) optical micrograph of adhering fungal cells stained with Giemsa; (**b**) invasion revealed by immunofluorescence microscopy; (**c**–**f**) SEM photomicrographs of internalized *P. brasiliensis*, (**d**, **e**): surrounded by vacuoles, (**f**) protoplast forms after 24 h of incubation. The arrows indicate the presence of *P. brasiliensis*

P. brasiliensis adhesion. The process seems to involve great changes in both the host cell and the fungal cytoskeletons [27, 29], suggesting that the capacity of the fungal cells to be internalized may be important in the development of the disease, even though *P. brasiliensis* is not an essentially intracellular parasite [32]. Thus, epithelial and endothelial cells could be a reservoir for the fungus, protected from the macrophages, as has been demonstrated in other diseases [10, 11]. The internalization of *P. brasiliensis* by epithelial cells could be a mechanism for the yeast cells to evade the professional phagocytes, which might help in the dissemination of the pathogen [19, 25]. Pulmonary epithelial cells are believed to play a crucial role in maintaining normal lung function. As the cells are strategically located at the interface between air and tissue, they primarily provide a morphological and functional barrier to the environment. In addition, airway epithelial cells clear the lung of extraneous material, while the alveolar

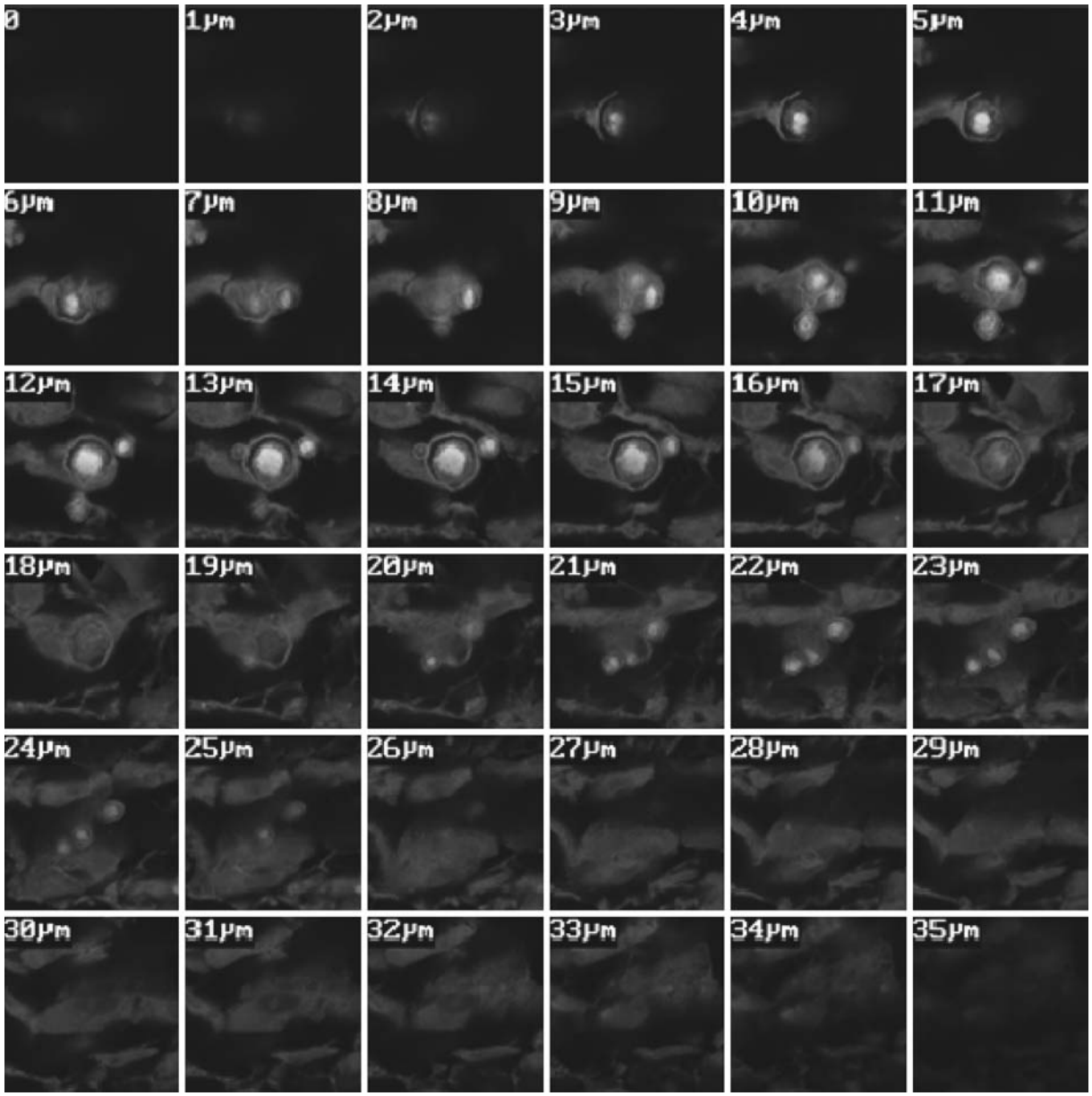

Fig. 2 Intracellular parasitism of *P. brasiliensis* in Vero cells after 5 h infection, shown in a laser confocal microscopy image gallery of 35 serial sections

epithelium is responsible for pulmonary gas exchange. However, recent data suggest that pulmonary epithelial cells may also modulate the inflammatory response to injurious agents [33].

On the other hand, migration of pathogenic yeasts to endothelial cells is considered a prerequisite for multiple organ invasion and dissemination of the fungus. We have investigated the adhesion of *P. brasiliensis* to endothelial cells, as such adhesion could represent a mechanism of dissemination. It was very hard to observe *P. brasiliensis* in the act of adhering to an endothelial cell monolayer, when examined periodically, suggesting that migration of the fungus across the endothelial layer is very fast, and cannot normally be observed in cell culture in vitro. Additionally, an experiment on the migration of

P. brasiliensis through an endothelial cell monolayer was carried out, and it was found that yeast cells migrated in greater numbers, and took less time, in control wells without cells. The fungus crossed the monolayer, but, the migration rate was about 30% lower than in control wells. This shows that the monolayer partially blocked migration of the fungus. Thus, *P. brasiliensis* can cross the endothelium rapidly, and probably invades deeper tissue. This model should be studied further to investigate the dissemination capacity of this fungus. During hematogenic dissemination, interactions of this nature constitute the first stages in the development of innumerable infections [34].

Adherence molecules

Molecules of adherence are fundamental in the pathogen-host interaction. These are usually exposed surface structures that facilitate adherence to host cells, or target host serum proteins of the extracellular matrix (ECM). During this interaction, the fungal cell wall is in continual contact with the host and acts as a sieve and reservoir of molecules such as adhesins [20, 35]. Our knowledge of the *P. brasiliensis* cell-surface structures and the basic mechanisms underlying their interaction with host receptor molecules has increased dramatically, through molecular and structural analysis of adherence molecules [20, 36].

Successful host tissue colonization by a fungus is a complex event, generally involving a ligand (adhesin) encoded by the pathogen and a cell receptor. The microorganism has the option of interacting with three types of host component: secreted cell products, host cell surface, or ECM proteins, such as types I and IV collagen, fibronectin, fibrinogen, and laminin. As described for bacteria [37], the search for new efficient treatments for systemic mycoses should be focused on the study of specific ligands or adhesins on the surface of the fungus that could adhere to various host substrata. Anti-adhesive drugs could then serve as a new way to fight infectious diseases.

Some of the receptors and ligands involved in the interaction of the fungus with the ECM have been identified at molecular level. The interactions of two samples of Pb18a (18a subcultured in PYG and 18b reisolated from infected hamster) were investigated with Vero cells and ECM proteins. Fungal cells bound to immobilized ECM proteins. Immunofluorescence labeling clearly demonstrated the presence of laminin, fibronectin, and type I and IV collagen binding sites on the surface of *P. brasiliensis* yeast cells. The reisolated sample (18b) demonstrated a higher capacity to bind to ECM proteins than the subcultured one (18a). Laminin was the most strongly bound component for both samples, followed by type I collagen, fibronectin, and type IV collagen for Pb18b. A remarkable difference was seen in the interaction of the two samples with fibronectin. Pb18a exhibited weaker binding to these ECM components. *P. brasiliensis* cell-free components (extracted from Pb18a or 18b) also adhered differently to 40 kDa and 120 kDa fibronectin fragments, Pb18b components interacting significantly with the 120 kDa fragment. Ligand affinity binding assays showed that type I collagen recognized two *P. brasiliensis* antigen components (47 and 80 kDa), and the major antigen gp43 bound both fibronectin and laminin [38].

This 43 kDa glycoprotein, a laminin ligand, plays a role in the adhesion [39]. This fact was confirmed when anti-gp43 serum prevented 85% of the *P. brasiliensis* from binding to Vero cells [28]. The gp43 peptide 1 (NLGRDAKRHL), with several positively-charged amino acids, contributed most to the adhesion of *P. brasiliensis* to Vero cells. Peptides RGDS (43.5%) in fibronectin, and CDPGYIGSR-NH2 (51.5%) and YIGSR (42.5%) in laminin showed the highest percentage inhibition of adhesion of gp43 to Vero cells [38].

Other fungal components also take part in the adhesion process [19]. Recently, a *P. brasiliensis* adhesin of 30 kDa was isolated with the capacity to bind to laminin. This protein was more expressed in the *P. brasiliensis* isolate that possessed a higher adhesion capacity. Treatment of a monolayer of epithelial cells with these two laminin adhesins (30 and 43 kDa) inhibited *P. brasiliensis* adhesion to the cells. Thus, a combination of these two adhesins significantly decreases the adhesion and invasion indices [40]. *P. brasiliensis* also shows two cell-surface proteins, of 32 and 19 kDa molecular mass that interact with several ECM proteins, such as laminin, fibronectin, and fibrinogen [41]. More recently, recombinant 3-glyceraldeyde phosphate dehydrogenase (GAPDH) from *P. brasiliensis* was found to be capable of binding to laminin, collagen I, and fibronectin. *P. brasiliensis* yeast forms treated

with anti-GAPDH were inhibited from infecting epithelial cells, and pneumocytes treated with recombinant GAPDH were resistant to fungal infection [36]. Paracoccin, a recently described adhesin, interacted with laminin in a dose-dependent manner. This interaction was inhibited by *N*-acetylglucosamine, followed by D-glucose and D-mannose, but not by D-galactose, *N*-acetylgalactosamine, or L-fucose [42]. Finally, ligand-affinity binding assays showed that a protein of 54 kDa (p*I* 5.6) had the properties of a fibronectin-binding adhesin [43]. Proteomic approaches will allow the characterization of adhesins produced under different conditions. Recently, we evaluated the Pb01 isolate before (Pb01A) and after (Pb01B) passage in epithelial cultured cells, in relation to protein expression, adhesion, and capacity of its cell-free extract to bind to laminin and fibronectin. We detected 197 spots in Pb01B extracts, 41 in Pb01A, and 19 matches. An increase in protein expression and the number of adhesins was evident in Pb01B after cell culture passage [44]. This occurrence could be associated with host adaptation and related to the virulence of *P. brasiliensis* [40, 45, 46]. Host-pathogen interactions reflect the balance of host defenses and pathogen virulence mechanisms. Advances in proteomic techniques now afford opportunities to compare protein content between complex biological systems ranging from cells to animals and clinical samples. A more in-depth study of host-pathogen interactions would improve our mechanistic understanding of pathogenicity and virulence, thereby defining novel therapeutic and vaccine targets.

The pathogen may regulate adhesin expression so as to survive and to produce illness. In *P. brasiliensis*, some adhesins have been described, and it is believed that all can play important roles in pathogenesis [20, 36, 39–41]. However, until now we have no *P. brasiliensis* strains with modified genes for these adhesins. Such strains have been described effectively for *Candida albicans* [47, 48], *C. glabrata* [49], *Blastomyces dermatitidis* [50], and *Coccidioides immitis* [51], and it was confirmed that adhesins play a role as virulence factors. *C. albicans* with genetically modified adhesins was incapable of adhering to epithelial and endothelial cells [47, 52]; equally, *B. dermatitidis* modified genetically in the main adhesin was unable to bind to pulmonary tissues [53, 54].

Invasion process

For many microbial pathogens, invasion of host cells is critical for the initiation and maintenance of infection, and many of these organisms have more than one mechanism to induce their own uptake by host cells [11, 55, 56]. Many of them can invade eukaryotic cells, and use this environment to multiply, to cross tissues and cause invasive disease, or to escape from the host immune response. Professional phagocytes and non-phagocytic cells, such as epithelial and normal endothelial cells, may be invaded [11, 57]. In many cases, cell invasion occurs because the microorganism induces its own uptake by these cells, specific extracellular signals stimulating cytoskeleton rearrangement at the point of contact with the microorganism. This rearrangement of cell microfilaments results in the endocytosis of the organism [58, 59]. Epithelial cells and fibroblasts do not encode CR3 receptors and, normally, they do not phagocytose. However, many intracellular pathogens are capable of entering these cells, by mechanisms involving integrins and cytoskeleton rearrangement. Bacterial pathogens manipulate the cytoskeleton of non-phagocytic eukaryotic cells to promote internalization, intracellular motility, and survival [60–62]. Some fungi that cause invasive disease, such as *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, invade host epithelial cells during mucosal and respiratory infection, and subsequently invade endothelial cells during hematogenous infection [11]. The pathogens can establish interactions with their hosts, and some have the ability to adhere, to invade, and to activate numerous signaling pathways. The study of the host-pathogen interaction supplies valuable data that help us to understand this diverse process. In *P. brasiliensis*, little is known about which pathways this fungus activates to survive and to escape from the monocyte-phagocyte system.

The invasion of A549 and Vero epithelial cells by *P. brasiliensis* has been shown to affect the cytoskeletal structure of the host cells, interfering in morphological aspects of the actin, tubulin, and cytokeratin components. Treatment with cytochalasin D and colchicine reduced the invasion index, indicating the functional participation of microfilaments and microtubules in this mechanism [22]. One plausible hypothesis is that *P. brasiliensis* expresses

at least two different mechanisms of invasion, one microfilament-dependent and the other microtubule-dependent. Expression of one or more fungal uptake pathways may depend on the presence of (so far unidentified) specific cell membrane receptors under particular environmental conditions and on the influence of other, surrounding cells. Why both pathways were observed in the same cell at the same time is at present hard to explain. However, the identity of any *P. brasiliensis* invasin or its epithelial receptor has not yet been discovered.

The fungus induces epithelial cells to produce pseudopodia (an actin-based movement of the cell surface) that engulf the organism and pull it into the cell. The formation of these pseudopodia is accompanied by the accumulation of epithelial cell microfilaments in the cytoplasm surrounding the organism. These microfilaments are required for endocytosis because disrupting them with cytochalasin D blocks this process. This mechanism is not used by all microorganisms, so it is considered as an important virulence factor. Some fungal species are known to be capable of invading mammalian cells in vitro and in vivi, but few studies have described the invasion process [11, 19, 22, 63–65].

On the other hand, the breakup of cytokeratin by *P. brasiliensis* may involve specific enzymes, possibly gp43 [66, 67], causing a loss of the filamentous structure. This degradation of cytokeratin could explain both the cell invasion process in vitro and the capacity of *P. brasiliensis* to cross epithelial barriers in vivi [10]. Perhaps the presence of specific fungal proteases plays an important role in the internalization and translocation of the fungus across epithelial barriers, as in *Candida albicans* [11].

The survival of *P. brasiliensis* in the intracellular environment may provide it with a protective mechanism against components involved in the immune response and the action of antifungal therapies. Microbial pathogens may enhance their ability to persist in infected hosts by causing the death of cells required for host defense. Although some intracellular pathogens may employ strategies to prevent cell death during pathogen replication, their subsequent escape and dissemination to new host cells may gradually require cell lysis. Apoptosis has important roles in organ development, cell differentiation, and the maintenance of homeostasis. However, it is speculated that there may be advantages for the host in the modulation of apoptosis, since non-professional phagocytes do not possess efficient microbicidal machinery, as do macrophages, and the death of these cells would not compromise the homeostasis of the organism. On the other hand, the death of cells with *P. brasiliensis* yeast cells inside could provide a route for dissemination to distant sites, such as a metastatic focus. Another hypothesis is that apoptotic cells with fungus inside would serve as a vehicle for gaining entry into macrophages without stimulating the microbicidal activities. If these ideas are correct, the succession of events in epithelial cells infected with *P. brasiliensis* in vitro would be fungal adhesion, translocation to the cell cytoplasm, multiplication, and induction of apoptosis. The correlation with pathogenic mechanisms in vivi could elucidate the initial steps of the infection [23]. By inducing apoptosis in macrophages, the microorganism could accomplish two goals: first, to kill the microbicidal cells in the tissues efficiently, and thus prevent its own death; second, to stimulate the inflammatory response and invade the tissues as a consequence of the damage caused by this response [68, 69].

We demonstrated that gp43 induced high levels of apoptosis in peripheral blood mononuclear cells [20]. In cultures of mononuclear cells from the peripheral system of patients with paracoccidioidomycosis, stimulated with gp43, IL-10 levels were found to be high and the addition of anti-IL-10 to these cultures increased the rate of apoptosis in cells stimulated only with gp43, suggesting an IL-10 anti-apoptotic role [70]. Studies in mice infected with *P. brasiliensis* showed that it can invade the thymus, inducing severe atrophy, caused by programmed cell death, suggesting that such alterations may be involved in the phenomenon of immunosuppression frequently associated with paracoccidioidomycosis [71]. Mendes-Giannini and collaborators [22] observed that *P. brasiliensis* induces apoptosis in epithelial cells. However, the fungus remained viable inside these cells, and multiplied. Expression of Bak and Bcl-2, the pro and anti-apoptosis signals, was not modified in the cells for up to 24 h of infection, suggesting a competition between survival and death mechanisms that allowed the infection to persist. High expression of Bak was observed after 48 h, indicating a loss of the competition by the survival signals. The mechanisms of invasion of host cells, persistence within them, and the subsequent induction of apoptosis of

such cells may explain the efficient dissemination of *P. brasiliensis*.

Many pathogens are capable of manipulating apoptosis in host cells, depending on the strain; the advantages of this manipulation may serve the pathogen or the host. Danelishvili et al. [72] have shown that *Mycobacterium tuberculosis* interacts with macrophages and epithelial cells in the lung alveolar space, where it is able to invade and multiply in these cells. Both virulent and attenuated strains of *M. tuberculosis* induced apoptosis in macrophages; however, the attenuated strain induced more apoptosis after 5 days of infection. In the epithelial cells, necrosis was observed more than apoptosis. Phagocyte apoptosis has been proposed as a strategy of the host to prevent the progress of the infection by causing the death of intracellular microorganisms. However, this can also allow the microorganisms to escape and infect neighboring cells, and spread to other tissues. *P. brasiliensis* is a more extracellular than intracellular pathogen [32, 73], and its escape to the intracellular compartment could favor its dissemination, while in histoplasmosis, fungus remains inside the apoptotic bodies and is destroyed [74]. Therefore, the mechanisms of invasion of the host cell, persistence inside the cell, and the subsequent induction of apoptosis of this same cell, may explain the efficient behavior of *P. brasiliensis* in promoting tissue infection and/or dissemination in the bloodstream. The invasion of normally non-phagocytic host cells can thus have different consequences, depending on the type of invading fungus. *Aspergillus fumigatus* blocks apoptosis of pulmonary epithelial cells [75], whereas *P. brasiliensis* induces apoptosis of epithelial cells [22], showing that the same host response can result in different parasite-host responses. One question not answered, refers to the fact that patients with paracoccidioidomycosis often present pulmonary fibrosis and exhibit severe respiratory limitations [76]. Fibrosis was attributed mainly to the progressive evolution of the granulomata toward cicatrization and to a lesser degree, probably, to direct induction by the fungus. Today, it is known that pulmonary fibrosis is characterized by the loss of lung epithelial cells and the proliferation of fibroblasts. In recent years, the topic of apoptosis in the lung has received a burst of attention from scientists fascinated by the cells of the vascular endothelium, epithelium, and immune system and interstitial cell populations. With regard to the epithelium, the initial demonstration by Fine et al. [77] that alveolar epithelial cells express functional Fas (CD95, APO1), was followed rapidly by the finding of Hagimoto et al. [78] that activation of Fas in vivo could induce epithelial apoptosis followed by fibrosis. The relationship between apoptosis and fibrosis in pulmonary paracoccidioidomycosis has not yet been studied.

Pathogens have developed a diversity of strategies to interact with host cells, manipulate their behavior, and thus, to survive and propagate [79]. During these processes, a great number of signaling pathways are activated. Using in silico search of the *P. brasiliensis* transcriptome-expressed sequence tag database for components of signaling pathways, several protein cascades in *P. brasiliensis* were described, such as (i) mitogen-activated protein kinase signaling for cell integrity, cell-wall construction, pheromone/mating and osmoregulation, (ii) the cAMP/PKA system, which regulates fungal development and virulence, (iii) the Ras protein, which allows cross-talk between cascades, (iv) calcium–calmodulin–calcineurin, which controls cell survival under oxidative stress, high temperature, and membrane/cell-wall perturbation, and (v) the target of the rapamycin pathway, controlling cell growth and proliferation. However, little is known on which pathway is activated to survive and to escape from the machinery of the host cells. Marques and co-workers [80] have identified four genes, RHO1, SEP1, FLB1, and PCK1, which encode proteins involved in cell signaling and polarity establishment in *P. brasiliensis*. Interestingly, RHO1 was expressed at 10- to 15-fold higher levels in minimal medium than in complete medium. De Carvalho and co-workers [81] reported that the Ca^{2+}/calmodulin signaling pathway has pleiotropic intracellular effects, acting on systems such as the cytoskeleton, and regulating nuclear transcription factors that may affect the expression level of other genes. In *P. brasiliensis*, this pathway could be involved in the regulation of genes differentially expressed in the yeast and mycelial forms. Single fragments were identified by Southern blot, suggesting that the *P. brasiliensis* calmodulin gene is probably a single copy, as in other fungi such as *H. capsulatum* [82] and *C. albicans* [83].

Recently, Monteiro da Silva et al. [84] observed that fungal invasion was significantly inhibited after

pretreatment of epithelial cells with genistein, a specific tyrosine kinase inhibitor, indicating that the tyrosine kinase pathway is involved in *P. brasiliensis* internalization. In contrast, when the fungus was treated, a slight (not significant) inhibition of PTK was observed, suggesting that PTK might not be the fungus' transduction signal pathway during the invasion process of epithelial cells. Intense PTK immunofluorescence labeling was observed at the border of the *P. brasiliensis* infected cells, and little PTK labeling was found in either uninfected cells or yeast cells, at later infection times (8 and 24 h). Moreover, when the epithelial cells were treated with genistein and then infected with *P. brasiliensis*, no labeling was observed, suggesting the importance of the PTK in the infectious process. These results suggest that the PTK pathway participates in the transduction signal during the initial events of the adhesion and invasion of mammalian epithelial cells by *P. brasiliensis*.

The signaling pathways involved in the cell proliferation, growth, and cytosol signals associated with the cytoskeleton during the invasion of epithelial cells by *P. brasiliensis* were mediated by actin rearrangement, and some virulence factors target the Rho family (Rho, Rac, and Cdc42), which are essential regulators of actin reorganization. Thus, the entry of *P. brasiliensis* into the epithelial cell can apparently require the activation of the small family of Rho GTPases, as demonstrated in a recent study [85]. In conclusion, in relation to this fungus, there is a need for more information on which proteins induce invasion, the host cell receptor, to which these endocytosis-inducing proteins bind, and the host cell signal transduction mechanisms that govern fungal invasion. However, we expect that the invasion mechanisms of *P. brasiliensis* will be more clearly elucidated in the near future.

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