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Recognition of laminin by *Paracoccidioides brasiliensis* conidia: a possible mechanism of adherence to human type II alveolar cells

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This study addresses the recognition of laminin by *Paracoccidioides brasiliensis* conidia, as well as its possible role in the adherence of conidia to A549 cells. Adherence of conidia to immobilized laminin was shown to be specific, as anti-laminin antibodies, soluble laminin or the laminin-derived peptides IKVAV and CDPGYIGSR inhibited this interaction. RGD containing peptides and various monosaccharides had no effect on adherence, with the exception of N-acetylneuraminic acid. Pre-treatment of conidia with fibrinogen and fibronectin, but not with BSA, also resulted in significant inhibition, suggesting that *P. brasiliensis* conidia might cross-recognize host proteins involved in colonization. In assays using transmission electron microscopy, we observed internalization of conidia 30 min after exposition to A549 cells. Laminin present on the surface of A549 cells shown to serve as mediator of this interaction, with a significant decrease in fungal adherence when the epithelial cells were pre-treated with anti-laminin antibodies or when conidia were pre-incubated with either soluble laminin or the laminin-specific peptides. Together these results suggest that the recognition of laminin by *P. brasiliensis* conidia is a key process in the interaction with pulmonary epithelial cells, where this extracellular matrix protein acts as bridging molecule.

Keywords *Paracoccidioides brasiliensis*, paracoccidioidomycosis, laminin, human type II alveolar cells, sialic acid

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis that is prevalent in most countries of Latin America. Initially the disease involves the lungs, with subsequent dissemination to other organs, including mucous membranes, skin, lymph nodes, and adrenal glands [1]. Infection is acquired by inhalation of the airborne conidia that are produced by the mycelial form of the fungus *Paracoccidioides brasiliensis*. These propagules are sufficiently small that they can reach the alveoli, whereupon they convert into yeast cells [2]. However, the mechanisms underlying the pathogenesis of PCM, or the means by which the fungus persists in the lungs and disseminates to other organs, have not been well described.

In the lungs, the basement membrane (BM) underlies cells of both the alveolar epithelium and the capillary endothelium, and it may become exposed after tissue damage. The BM is a specialized type of extracellular matrix (ECM); its composition is known to include laminin, a heterotrimeric glycoprotein composed of alpha, beta, and gamma chains held together by disulfide bonds, which play an important role in cell adhesion, proliferation, and differentiation [3].

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Several fungi of clinical importance, such as *Candida albicans* [4], *Aspergillus fumigatus* [5], *Histoplasma capsulatum* [6], *Pneumocystis carinii* [7], and *Penicillium marneffei* [8] are known to interact with laminin. Interactions between laminin and *P. brasiliensis* have been described before [9–14]. In particular, in a previous study our group demonstrated the presence of two proteins of 19 and 32 kDa on the surface of *P. brasiliensis*, which recognized not only laminin but also fibronectin and fibrinogen. In that study, a monoclonal antibody raised against the 32 kDa protein inhibited the adherence of *P. brasiliensis* conidia to these immobilized proteins, indicating that such interaction is mediated at least partly by laminin, as well as by fibronectin and fibrinogen [13]. In the present communication, we address the nature of the molecules involved in the interaction between *P. brasiliensis* conidia and laminin.

Previously, it was shown that laminin increased the severity of lesions in a hamster testicle infection model and enhanced the adhesion of yeast cells to both MDCK and A549 epithelial cell lines [11,12]. In the experimental conditions of the present work pre-treatment of conidia with soluble laminin inhibited their adherence to A549 cells.

Our findings indicate that sialic acid and the laminin binding motifs IKVAV and YIGSR mediate the recognition of this key lung component, with laminin also participating as bridging molecule in the interaction of conidia with A549 lung cells. These results provide further evidence for the interaction of *P. brasiliensis* conidia with lung components, and suggest a possible role for laminin in the early establishment of the disease.

Materials and methods

Reagents

Laminin (derived from Ebgelbreth-Holm Swarm mouse sarcoma), immunochemicals and reagents employed in this study were obtained from Sigma Chemical Co. (Poole, Dorset, UK), unless otherwise specified.

Conidial isolation

Paracoccidioides brasiliensis strain Gra (ATCC No. 60855) was used in the study. The fungus was maintained in its mycelial form and cultures were expanded in liquid medium, prior to induction of spore formation, as reported previously [15]. Conidia to be used in adherence assays were obtained by the discontinuous Percoll gradients method [16]. Briefly, cultures were flooded with 0.01% (v/v) Tween 20 in saline solution, fungal growth was scraped, and conidial suspensions were fragmented by shaking with glass beads (250 rpm), centrifuged, and the pellet sonicated at 7 Hertz for 15 sec. The fungal material obtained from each Petri dish was layered over the Percoll gradient (95% in 0.25M sucrose), centrifuged, and the pellet (consisting of detached conidia) was retrieved and washed twice with phosphate-buffered saline (PBS). Conidia were counted in a hemocytometer, tested for viability using the fluorescein-ethidium bromide technique [17] and checked for mold and bacterial contamination.

Labeling of Paracoccidioides brasiliensis *conidia*

In order to study adherence to immobilized laminin, 1×10^7 conidia/ml, previously incubated with PBS containing 1% (v/v) H_2O_2 for 15 min, were labeled with biotin and incubated with streptavidin peroxidase, as previously described by Peñalver *et al*. [18]. Additionally, when assessing the adherence of *P. brasiliensis* conidia to A549 cells, propagules were labeled with fluorescein by first resuspending 1×10^7 conidia in 1 ml of 0.1M carbonate buffer (pH 9.0) containing 0.1 mg/ml of fluorescein isothiocyanate (FITC), and incubating for 1 h at room temperature in the dark. FITC-labeled *P. brasiliensis* conidia were washed twice with PBS containing 0.25% of BSA and re-suspended to an appropriate concentration in Minimum Essential Medium with Earle's salts (EMEM) (Gibco BRL).

Adherence of Paracoccidioides brasiliensis *conidia to immobilized laminin*

As described elsewhere [8,18], 96-well microtiter plates (Maxisorp; Nunc A/S, Kamstrup-Denmark) were initially coated with a range of different concentrations of laminin (0.01–100 μg/ml) dissolved in PBS, and incubated overnight at 4°C and for 1 h at 37°C. Plates were then blocked with 1% (w/v) BSA solution in PBS (1 h, 37°C). Biotin labeled conidia were added in appropriate quantities (5×10^4 conidia/well) and incubated for 1 h at 37°C. Non-adherent fungal cells were removed by washing with PBS containing 0.05% (v/v) Tween-20. In other experiments, plates were coated with 50 μg/ml of laminin and a range of biotin labeled conidia (from 5×10^3 to 5×10^4 conidia per well) were added. Wells coated with 1% BSA in PBS were used throughout as a negative control. Color reaction

was developed using 3,3′,5,5′-Tetramethylbenzidine (BD Biosciences, CA, USA), and intensity was determined at 450 nm with an automated plate reader. Results were expressed as absorbance at 450 nm (A_{450}), and represent the means ± SEM of two or three independent experiments.

Specificity of binding to immobilized laminin and inhibition experiments

To address the specificity of laminin recognition by conidia, coated plates were pre-incubated for 1 h at 37°C with rabbit anti-laminin polyclonal antibodies or with an irrelevant antibody raised in the same species (sc-651, Santa Cruz-Biotechnology, CA, USA) made up in PBS. We also investigated the effect of pre-incubation of labeled conidia (2 h, 37°C) with soluble laminin and the laminin specific peptides (YIGSR, CDPGYIGSR and IKVAV) at a final concentration of 1 mg/ml. For the inhibition experiments biotin labeled conidia were pre-treated for 2 h at 37°C, prior to their addition to the standard adherence assay, with soluble proteins (BSA, fibrinogen, fibronectin and RGD peptides at 1 mg/ml); with sugars such as D(+)mannose, D(+)glucose, D(+)galactose, N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), N-acetylneuraminic acid (NANA), at a final concentration of 200 mM; or with sialoglycoproteins (mucine and asialomucine at 200 μg/ml). Untreated conidia were used as positive controls. As a reference, the A_{450} values of the positive controls were taken as 100% adherence; results are presented as percentage absorbance of the reference values (mean ± SEM).

Influence of enzymatic treatment of conidia on laminin adherence

To determine the effect of proteolysis, conidia were pre-incubated for 1 h at 37°C with α-chymotrypsin or trypsin made up in PBS at a final concentration of 1000 μg/ml [19] and the reaction was stopped by the addition of 100 mM phenyl methyl sulphonyl fluoride (PMSF) or 10% (v/v) foetal calf serum, respectively. In other experiments the importance of cell wall integrity on adherence was examined by treating conidia for 1 h with chitinase 1000 μg/ml made up in 50 mM Tris-HCl, pH 8.0 at 37°C or with neuraminidase from *Vibrio cholerae* at 0.4 U/ml in PBS pH 6.0 (0.01M phosphate buffer, 0.15M NaCl) [20]. Conidial suspensions were incubated for 30 min at 37°C, the enzymatically-treated fungal cells were then pelleted and after two further washes in PBS they were tested in the standard adherence assay. Controls consisted of conidia pre-incubated with PBS only.

Detection of laminin on the surface of A549 cells

The human lung epithelial cell line A549 was obtained from the European Collection of Cell Cultures (ECACC) and grown as specified. Sub-confluent A549 cells were harvested by 0.25% trypsin treatment and re-suspended at a concentration of 1×10^6 cells in PBS containing rabbit anti-laminin antibodies (1:50). After incubation at room temperature for 1 h with shaking, cells were washed and re-suspended in PBS containing a goat anti-rabbit FITC-conjugated antibody and incubated for 1 h at room temperature. Cells were then washed, re-suspended in PBS-formaldehyde 4%-sodium azide 1% and analyzed by flow cytometry using an EPICS XL (Coulter Electronics, Inc., Hialeah, FLA). Data were analyzed using WinMDI 2.8. As a negative control, equal numbers of cells were processed in the absence of anti-laminin antibodies but in the presence of PBS or of an isotype control.

Adherence of P. brasiliensis *conidia to A549 cells*

The assessment of the early interaction between *P. brasiliensis* conidia and the lung cells were performed. Confluent monolayers were obtained by seeding 5×10^4 cells per well using LabTek® 8 well slides (Nunc, Kamstrup, Denmark) and incubated for 24 h. Cell monolayers were washed once and viable conidia (obtained via the discontinuous Percoll gradient method) were added at a concentration of 5×10^4 conidia per well. Co-cultures were then incubated for 0.25, 0.5, 1, 2, 3, and 24 h at 37°C in 5% CO_2. At the end of the incubation period, the monolayers were washed twice with culture media without FBS, fixed for 2 h at 4°C with 2.5% (v/v) glutaraldehyde in 10 mM phosphate buffer and processed for standard transmission electron microscopy (TEM) procedures.

In other experiments, confluent monolayers were obtained by adding 5×10^4 cells per well to 96-well tissue culture plates (Nunc, Kamstrup, Denmark) and incubating them for 24 h. Then 25×10^4 FITC-labeled conidia were added to the A549 cells and incubated for 3 h at 37°C in 5% CO_2, and co-cultures were then washed, harvested and analyzed by flow cytometry as described above. The number of fluorescent-positive A549 cells was taken as the percentage of adherence. In addition, the participation of laminin in the adherence to A549 cells was addressed. To this end, FITC-labelled conidia were pre-incubated for 2 h with soluble laminin

(100 μg/ml) or laminin derived peptides (1 mg/ml), and added to A549 cells. Additionally, the effect of anti-laminin antibodies (1:10) or of an isotype control on adherence to pulmonary epithelial cells was also determined as previously described [4]. The A549 cells were kept for 1 h, washed twice and then exposed to the FITC-labelled conidia (25×10^4). Co-cultures were incubated for 3 h, after which the cells were washed and analyzed by flow cytometry as described. Untreated conidia or BSA-treated conidia were used as controls. Inhibition of the adherence was calculated using the following formula:% inhibition $=(1-(A/B)) \times 100$, where A was the mean number of conidia adhering to treated cells and B was the mean number of conidia adhering to untreated cells, or where A was the mean number of treated conidia adhering to A549 cells and B was the mean number of untreated conidia adhering to A549 cells.

Statistical analysis

Results are expressed as the mean ± SEM of two or three independent experiments run in triplicate. Data were analyzed by one-way ANOVA and Student's *t*-test using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com).

Results

Adherence of conidia to immobilized laminin

Incubation of conidia with increasing concentrations of laminin (0.01–100 μg/ml) resulted in a progressive increase of adherence, with apparent saturation being reached at a concentration of 50 μg/ml (Data not shown). Adherence to laminin at any given concentration depended on the number of conidia added to the coated wells. Thus at a laminin concentration of 50 μg/ml, adherence rose with increasing conidial concentration, and the maximal absorbance was reached at 5×10^4 conidia (Data not shown). Accordingly, a concentration of 50 μg/ml of laminin and 5×10^4 conidia per well were selected for all subsequent experiments using this adherence assay. Labeling with the biotin-streptavidin-peroxidase complex did not affect the ability of conidia to interact with the immobilized ligand, as determined by the microscopic counting of unlabeled and labeled conidia attached to coated wells (data not shown).

Specificity of binding to laminin

The addition of the anti-laminin polyclonal antibody at 1:100 and 1:1000 dilutions resulted in a statistically significant inhibition ($p < 0.001$) of the adherence of *P. brasiliensis* conidia to immobilized laminin (Data not shown), when compared to the PBS-treated control. This effect was also statistically significant when compared to the effect of an irrelevant antibody, and depended on the antibody concentration ($p < 0.001$ and $p < 0.05$ for the dilutions 1:100 and 1:1000, respectively). At higher dilutions the anti-laminin antibody was no longer inhibitory. The pre-incubation of conidia with soluble laminin caused a statistically significant ($p < 0.01$) decline in their adherence to the immobilized protein, as did the treatment with the laminin derived peptides CDPGYIGSR and IKVAV ($p < 0.05$ and $p < 0.001$, respectively; Fig. 1). The presence of soluble BSA (1 mg/ml) did not affect the ability of conidia to interact with immobilized laminin, but the addition of fibrinogen and fibronectin had an inhibitory effect that was greater than that of laminin ($p < 0.001$; Fig. 1). In contrast, the short peptides (RGD, RGDS and YIGSR) had no discernable effect on the binding of conidia to immobilized laminin (Fig. 1).

Influence of sugars in Paracoccidioides brasiliensis conidia adherence

The adherence of conidia to immobilized laminin was independent of monosaccharides, mucin and

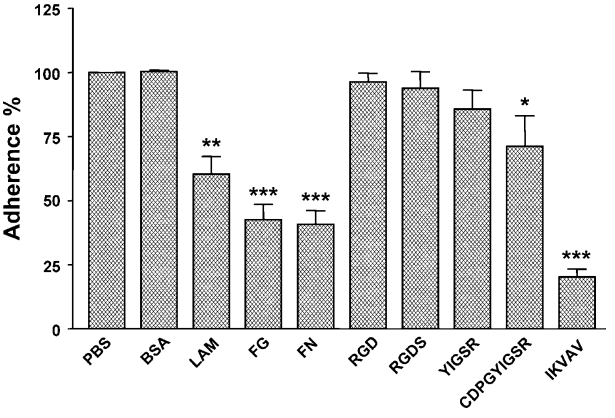

Fig. 1 Inhibition of the adherence of *Paracoccidioides brasiliensis* conidia to laminin. In order to determine the effect of inhibition by soluble ligands, wells were coated with laminin and conidia were allowed to adhere without any inhibitor present, or after pre-incubation with soluble fibronectin (FN), soluble fibrinogen (FG), soluble laminin (LAM), RGD, RGDS YIGSR, CPDGYISR, and IKVAV peptides, all at a final concentration of 1 mg/ml (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus PBS control). Results are expressed as the mean of the percentage absorbance of the reference values ± SEM of two or three independent experiments.

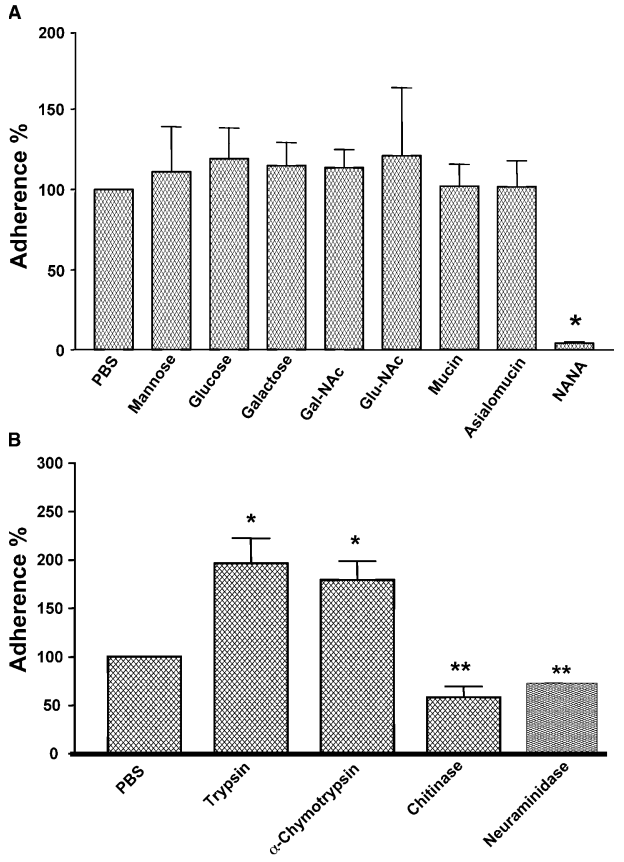

Fig. 2 Influence of sugars on the adherence of *Paracoccidioides brasiliensis* conidia to laminin. The contribution of monosaccharides was addressed by pre-incubating conidia with D(+)mannose, D(+)glucose, D(+)galactose, N-acetyl-D-galactosamine (GalNAc) or N-acetyl-D-glucosamine (GlcNAc), mucin, N-acetyl-neuraminic acid (NANA), or asialomucin, in PBS; *, $p < 0.001$ versus PBS control (A). To determine the effect of enzymatic treatment on the interaction between conidia and immobilized laminin, *Paracoccidioides brasiliensis* conidia were treated with trypsin, α-chymotrypsin and chitinase at a final concentration of 1000 μg/ml, or with neuraminidase from *Vibrio cholerae* (0.4 U/ml) (B). The enzymatic treated cells were tested in the standard adherence assay (*, $p < 0.05$; **, $p < 0.01$ versus PBS control). Results are presented as the mean of the percentage absorbance of the reference values ± SEM of two or three independent experiments.

asialomucin. In contrast, the presence of NANA and the neuraminidase treatment of conidia had a significant inhibitory effect ($p < 0.001$ and $p < 0.01$, respectively; Fig. 2A and 2B).

Influence of the enzymatic treatment of conidia on adherence to laminin

The treatment of conidia with trypsin and α-chymotrypsin increased *P. brasiliensis* conidia adherence ($p < 0.05$; Fig. 2B). In contrast, chitinase treatment (Fig. 2B) significantly inhibited their adherence to the immobilized protein ($p < 0.01$), indicating the involvement of cell wall structures in the adhesion process.

In vitro *interaction between conidia and type II alveolar cells*

TEM studies revealed that as early as 30 min after exposure, *P. brasiliensis* conidia could be observed intracellularly (Fig. 3), although no special disposition of cellular organelles were observed. The internalized conidia appeared to be localized in the proximity of the nucleus.

Participation of laminin in adherence of Paracoccidioides brasiliensis *conidia to human type II alveolar cells*

Assays were performed to determine whether laminin was present on the surface of A549 cells, with the aim of establishing its role in mediating the adherence of *P. brasiliensis* conidia to the cell line. We observed that when A549 cells were exposed to the anti-laminin antibodies they attached to the cells, indicating the presence of this ECM protein on the surface of A549 cells (Data not shown).

Once the presence of laminin on the surface of A549 cells was established, we next investigated whether anti-laminin antibodies, the soluble protein, or laminin-derived peptides would interfere with conidial adherence to these cells. When compared to controls, treatment of A549 monolayers with anti-laminin antibodies showed an inhibitory effect on the adherence of conidia (40.2%, $p < 0.0001$; Fig. 4A). Similarly, pre-treatment of conidia with the soluble protein or the specific peptides had a significant inhibitory effect ($p < 0.05$) on the adherence to A549 cells when compared to the controls (Fig. 4B and 4C), although the highest inhibitory effect was observed in the presence of soluble laminin (53.7%, $p < 0.001$).

In addition, when adherence was assessed in the presence of EMEM complete culture medium, similar results were observed (data no shown), suggesting that the minimal concentrations of plasma proteins contained in FBS had not contributed to this interaction.

Discussion

In this communication, the interaction of laminin with *P. brasiliensis* conidia is described. Plate adherence assays demonstrated that these propagules were able to bind to immobilized laminin, and that this interaction was dependent on the concentration of laminin and the conidia, with increasing concentrations of protein or conidia resulting in progressively higher adherence.

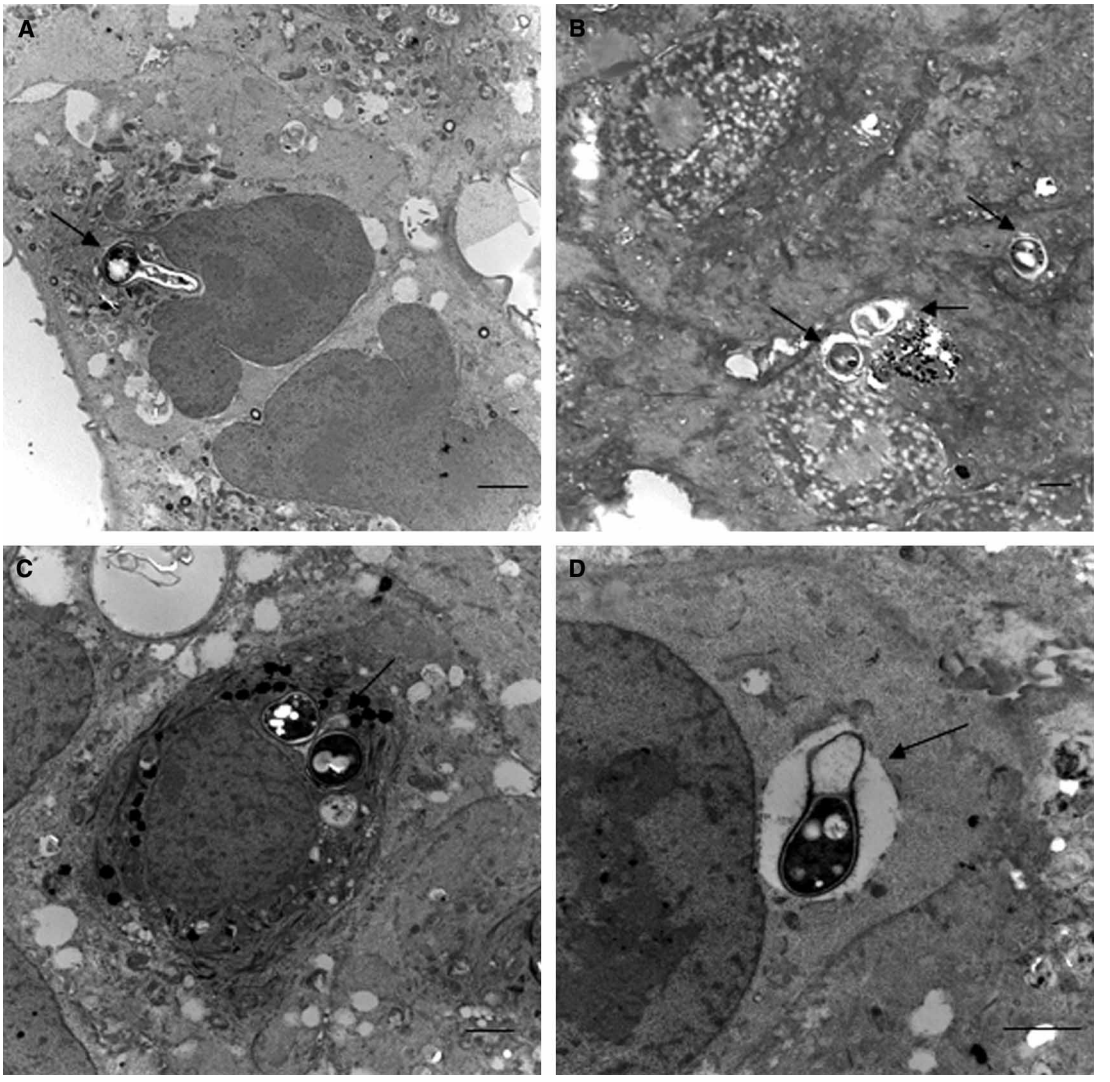

Fig. 3 Transmission electron micrographs of the type II alveolar cells after incubation with *Paracoccidioides brasiliensis* conidia. (A) Conidium internalized after 30 min of exposure. Magnification ×5,000. (B and C) Conidia inside type II alveolar cells after 3 h and 24 h of incubation, respectively. Magnification ×2500 and ×7000, respectively. (D) Conidium internalized in a membrane-bound vacuole, after 24 h of co-culture. Magnification ×10,000. Bar =2 mm. The arrows indicate the intracellular conidia.

As might be expected in a receptor-ligand mediated recognition process, both soluble laminin and anti-laminin antibodies were able to inhibit the binding of conidia to immobilized laminin, indicating the specificity of this process. Interaction also involved amino acid sequences such as YIGSR, located on the β1 chain, and IKVAV, located on the COOH-terminal end of the α chain, which are exclusive of the laminin molecule. Both peptides have been shown to be involved in cell adhesion and migration, and to inhibit or promote angiogenesis and tumor metastasis, respectively [21,22]. Pre-treatment of *P. brasiliensis* conidia with IKVAV peptides resulted in a significant inhibition of adherence. This observation parallels a previous finding with *H. capsulatum* yeast cells concerning laminin binding [6], a process that is also dependent on the IKVAV sequence. In addition, the CDPGYIGSR peptide also caused inhibition of conidial binding, although to a lesser extent than the IKVAV peptide. Interestingly, the YIGSR peptide, which has been reported to inhibit *Sporothrix schenckii* binding to laminin [23], did not have any significant effect on the adherence *of P. brasiliensis* conidia to the immobilized protein. However, it efficiently

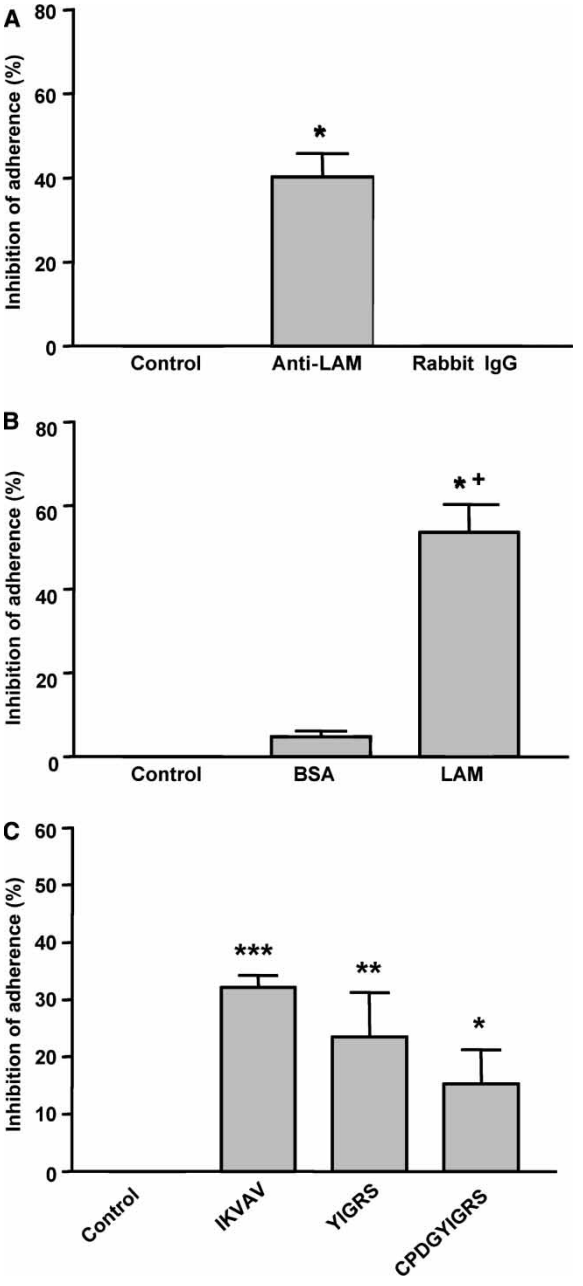

Fig. 4 Participation of laminin in the adherence of *Paracoccidioides brasiliensis* conidia to A549 cells. The effect of treatment of A549 cells with antibodies against laminin (1:50 dilution) (A), and of *Paracoccidioides brasiliensis* conidia with soluble laminin (100 μg/ml) (B) or laminin derived peptides YIGSR, CPDGYISR, and IKVAV (1 mg/ml) (C) was assessed. The values represent the mean of the adherence inhibition in percentage ± SEM. Significant differences when compared to the PBS control (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$) or versus BSA ($p < 0.01$; +) are indicated.

inhibited their interaction with the A549 cells, suggesting that the conformation of laminin and laminin-derived peptides might alter their binding specificity [24].

Fibronectin and fibrinogen, but not BSA, were also able to inhibit the interaction with immobilized laminin. It is known that pathogen-host interactions are complex and redundant, and this cross inhibition phenomenon strongly suggests that binding of *P. brasiliensis* conidia to ECM proteins could be mediated by common mechanisms, as indicated previously [13,25]. ECM proteins are known to possess N-linked sialic acid residues at the end of oligosaccharide chains [26,27], and it seems likely that the cross inhibition observed when *P. brasiliensis* conidia were pre-incubated with other soluble ECM proteins arose from the presence of this shared motif. We recently demonstrated that NANA was the only tested monosaccharide able to significantly inhibit adherence to laminin (this paper) and to other ECM proteins [25]. Exposed sialic acids residues contribute significantly to the structural properties of the molecules, so it is not surprising that sialic acids posses an important role as regulators of cellular and molecular interactions [28]. The presence of sialic acid has been reported in opportunistic and pathogenic fungi and its participation in the recognition of ECM proteins has been demonstrated for *A. fumigatus*, *P. marneffei*, and *S. schenckii* [19,23,26,29]. In *P. brasiliensis*, sialic acid has been detected in both mycelia and yeast forms, where it represents the only anionogenic group detected to date [20]. Although its biological role has not been elucidated, our present results suggest that sialic acid on the fungus cell wall or in the laminin molecules themselves might be involved in the interaction, as pre-treatment of conidia with neuraminidase or NANA resulted in inhibition. It remains to be established if sialic acid residues account for this interaction, by simply contributing to electrostatic interactions as has been suggested for *A. fumigatus,* with the binding of conidia to ECM proteins being mediated by ionic interactions, probably involving the GAG binding domains present in the ECM proteins [30], or if this process involves a more refined recognition mechanism.

In the other hand, it is noteworthy the lack of inhibition by mucine or its desyalilated form, asialomucin, suggesting that other mechanism might also being involved in this interaction. Sialic acid residues present in laminin and in other ECM proteins are N-linked, while in mucins they are O-linked. Therefore, their structural conformation may well influence recognition. Sialic acid lectins are known to be highly specific as illustrated by the influenza virus hemagglutinin [31]. To date, however, no sialic acid lectin has been described for *P. brasiliensis.*

In addition to sialic acid, ECM proteins share other motifs, one of the most important is its being in the

RGD domain that represents the vertebrate integrin recognition sequence. This tripeptide is also recognized by the integrin-like receptors described in pathogenic fungi such as *C. albicans*, *C. tropicalis* and *A. fumigatus* [5,32]. Inhibition assays showed that the ability of *P. brasiliensis* conidia to interact with immobilized laminin was unaffected by the addition of the RGD peptides, suggesting that the interaction between laminin and conidia is not mediated by integrin-like receptors.

The ability of conidia to interact with immobilized laminin was significantly improved by treating them with α-chymotrypsin and trypsin. This finding appears consistent with the presence on the conidial surface of cryptic receptors, referred to as 'cryptitopes'. These cryptic receptors correspond to masked molecules or hidden segments that may become exposed as a result of enzymatic action, or represent molecules that have undergone conformational changes [33]. However, in *P. brasiliensis,* these molecules are currently of unknown biochemical nature.

It has recently been shown that some cell wall components may play an essential role in virulence, not only by maintaining the structural integrity of the cell wall, but also via the binding of molecules involved in the interaction with host tissues [23,34,35]. In our study, treatment of conidia with chitinase significantly reduced their ability to interact with laminin, although the addition of N-acetyl-glucosamine, the main subunit of the chitin polymer, did not inhibit interaction, suggesting that direct recognition of chitin might not be sufficient to explain the inhibition observed. It remains to be elucidated if chitin mediates the interaction of *P. brasiliensis* conidia with laminin by linking a particular adhesin to the cell wall, as has been described for *Blastomyces dermatitidis* (WI-1, BAD-1) and *H. capsulatum* (YpS3) virulence factors [34–36].

Adherence and invasion of type II alveolar cells are important in the initial stages of lung colonization by several pathogens and might also play a role in the persistence of the pathogen in the host [37–39]. Adhesion of *P. brasiliensis* yeasts to type II alveolar cells, as well as the internalization of pathogenic bacteria and opportunistic fungi by A549 cells has been shown previously [37,39–41]. However, this is the first report of adhesion and internalization of conidia from a dimorphic fungus, with conidia considered to be the most important *P. brasiliensis* infecting propagules. The present data suggest that the up-take of conidia involves the participation of ECM proteins as bridging molecules between conidia and mammal cells. It remains to be established if conidia become yeast cells, replicate inside A549 cells, or whether these cells exert some activity that may affect conidia viability.

We found that antibodies against laminin, or laminin in its soluble form, significantly inhibited the adherence of conidia to these lung epithelial cells, indicating that laminin is involved in the interaction with the fungal propagules. Similarly, we observed that pre-treatment of conidia with specific laminin motifs (IKVAV and YIGSR) significantly inhibited the adhesion to type II alveolar cells. In addition, we recently observed that the addition of sialic acid to the cell system inhibited almost completely the adhesion of *P. brasiliensis* conidia to A549 cells [25].

Recently, André *et al*. [42] showed that pre-treatment of *P. brasiliensis* yeast cells with laminin led to a less severe pathologic process in a pulmonary mouse model of infection induced by virulent and low-virulence isolates. These investigators speculated that pre-treatment might have covered laminin binding epitopes, thus preventing their *in vivo* interaction with the corresponding molecules and resulting in lower adherence of fungal cells. In our present study, if one considers that laminin was detected on the surface of A549 cells, the observed reduction of adherence in the presence of laminin antibodies might be the result of stearic hindrance, whilst the soluble protein and the peptides could act by covering the adhesins present on the external layer of the fungus, thus hindering its interaction with laminin present on the epithelial surface.

On the other hand, it is known that type II alveolar cells secrete surfactants which are lipoprotein complexes found in the fluid lining the alveolar surface of the lungs. Since surfactant covers the pulmonary surface, it may well act as a protective layer in charge of inhibiting the binding of *P. brasiliensis* conidia to all of the pulmonary cells. Since *P. brasiliensis* conidia bound efficiently to the A549 type II alveolar epithelial cells, which secrete surfactant, the latter possibility appeared unlikely. By the same token, similar results were described by Galvan *et al*. [43], who observed that surfactant did not inhibit *Yersinia pestis* binding to A549 cells. These findings appear highly relevant to the study of potential interactions among *P. brasiliensis* conidia and surfactant.

In conclusion, these findings indicates that *P. brasiliensis* conidia interact specifically with laminin and type II alveolar cells, and suggest that the recognition process of the latter could involve the participation of host proteins, such as laminin, acting as bridging molecules between conidia and host cells.

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