

The influence of carbohydrates in the interaction of *Paracoccidioides brasiliensis* with CCL-6 cells *in vitro*

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ABSTRACT

Introduction: Little is known about the early events in the interaction between *Paracoccidioides brasiliensis* and its host. To understand the effect of carbohydrates in the interaction between the fungus and epithelial cell in culture, we analyzed the influence of different carbohydrate solutions on the adhesion of *P. brasiliensis* yeast cells to CCL-6 cells in culture. **Methods:** Fungal cells were cultivated with the epithelial cell line, and different concentrations of D-fucose, N-acetyl-glucosamine, D-mannose, D-glucosamine, D-galactosamine, sorbitol and fructose were added at the beginning of the experiment. Six hours after the treatment, the cells were fixed and observed by light microscopy. The number of *P. brasiliensis* cells that were adhered to the CCL-6 monolayer was estimated. **Results:** The number of adhesion events was diminished following treatments with D-fucose, N-acetyl-glucosamine, D-mannose, D-glucosamine and D-galactosamine as compared to the untreated controls. Sorbitol and fructose-treated cells had the same adhesion behavior as the observed in the control. *P. brasiliensis* propagules were treated with fluorescent lectins. The FITC-labeled lectins WGA and Con-A bound to *P. brasiliensis* yeast cells, while SBA and PNA did not. **Conclusions:** The perceptual of adhesion between *P. brasiliensis* and CCL-6 cells decreased with the use of D-mannose, N-acetyl-glucosamine and D-glucosamine. The assay using FITC-labeled lectins suggests the presence of N-acetyl-glucosamine, α -mannose and α -glucose on the *P. brasiliensis* cell surface. An enhanced knowledge of the mediators of adhesion on *P. brasiliensis* could be useful in the future for the development of more efficient and less harmful methods for disease treatment and control.

Keywords: Paracoccidioidomycosis. Adhesion. CCL-6 cells. Lectin labeling.

INTRODUCTION

Paracoccidioidomycosis (PCM) is a disease endemic in Latin America that is caused by a thermo-dimorphic fungus, *Paracoccidioides brasiliensis*^{1,2}. The fungi initially infect the lungs, but can also disseminate throughout the entire body³⁻⁵. *Paracoccidioides brasiliensis* is a biotrophic pathogen, and an acute and sub-acute clinical form of PCM can be observed in patients³. Unfortunately, little is known about the ecological niche of the saprophytic phase of *P. brasiliensis* and its modes of infection and dissemination throughout the host. The cellular events that lead to infection are also unclear, but undoubtedly the disease is established with the conversion of the filamentous phase of the pathogen into its yeast phase, followed by the adhesion of the pathogenic yeast form to the hosts tissue and fungal multiplication⁵.

The adhesion event is better characterized in other pathogenic fungi, and there are only a few reports concerning the precise mechanism of binding between *P. brasiliensis* and its host. Most pathogenic fungi express surface factors that mediate the direct or indirect binding to host cells. For the latter, binding to the host occurs through the interaction with adhesion components, such as extracellular matrix (ECM) proteins, which act as interlinking molecules^{6,7}. For a number of pathogens, the ability to bind ECM glycoproteins and the capability of internalization are considered to be

important characteristics, and molecules, such as laminin, fibronectin, integrin and carbohydrates, mediate these events⁶.

The diagnostic glycoprotein gp₄₃, which is present on the *P. brasiliensis* cell wall, has binds to laminin⁸. Other fungi, such as *Aspergillus fumigatus*, also bind to the laminin molecule⁹. Another molecule that is associated with the interaction between the fungus and host is fibronectin. The participation of this molecule in the establishment of the host/pathogen interaction is evident in *Candida albicans*¹⁰. Laminin and fibronectin normally interact with integrins. In fungi, the presence of integrin-like proteins was characterized in *C. albicans* and *Uromyces appendiculatus*^{10,11}. The regulation of cell adhesion by fungal surface carbohydrates has been characterized in *Fonsecaea pedrosoi* and *Trichophyton mentagrophytes* conidia during the initial stages of interaction with host cells^{12,13}. However, little is known about the mechanism of carbohydrate-mediated adhesion in fungi.

In vitro studies are a helpful in the characterization of adhesion events and mediators. The adhesion *P. brasiliensis* to cultured cells has been previously reported^{6,14}. Vero, LLC-MK₂ and Henle-407 epithelial cell lines are able to support *P. brasiliensis* adhesion and infection¹⁵. It has been shown any evidence of the presence of mannose 43kDa glycoprotein (gp43) of *P. brasiliensis* and also their property of binding to Con A¹⁶. To study the role of monosaccharides in the adhesion of *P. brasiliensis* to its host, fungal propagules were cultivated with CCL-6 cells with and without the presence of sorbitol, D-mannose, D-fucose, N-acetyl-glucosamine, D-glucosamine, fructose and D-galactosamine solutions. The number of adhesion events was estimated. The lectins wheat germ agglutinin (WGA), *Concanavalia eosiformes* agglutinin (Con-A), soybean-*Glycine max* agglutinin (SBA) and peanut-*Arachys hypogaea* agglutinin (PNA) were conjugated to fluorescein isothiocyanate (FITC) and used to detect the presence of carbohydrates on the *P. brasiliensis* surface.

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METHODS

Microorganism

The *P. brasiliensis* (Pb₁₈) strain was obtained from the Mycology Laboratory Collection in the Biological Sciences Institute at the Federal University of Minas Gerais, Minas Gerais, Brazil (ICB-UFMG) and maintained in its leveduriform state on partially solid Fava-Netto culture medium¹⁷ at 37°C. Prior to cellular interactions, the fungi were adapted to Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich, St. Louis MO-USA) tissue culture medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis MO-USA) and antibiotic solution (Gentamicin 60mg/L - Ariston - Brazil) under agitation (130rpm) at 37°C for 7 days. After incubation, the cell viability was estimated using a FDA/EB (fluorescein diacetate 2.5mg/ml + ethidium bromide 20µg/ml) solution for 30min; the green fluorescent cells were considered viable (Olympus BX-41 microscope - WB filter)¹⁸.

Host cell line

The host cell line (ATCC CCL-6) was kindly provided by Dr. Judith A. Apletton (Veterinary Medicine School, Cornell University - USA) and maintained in RPMI-1640 (Sigma-Aldrich, St. Louis MO-USA) in 25-ml flasks, supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis MO-USA) and antibiotic solution (gentamicin 60mg/L - Ariston). The cells were incubated at 37°C in an atmosphere containing 5% CO₂. Cell viability was estimated using a 0.4% Trypan blue solution and the non-stained cells were considered viable. Only cultures with more than 90% viability were utilized in the infection assays. All experiments were performed with cells obtained from the second passage in RPMI culture medium¹⁹.

Host/pathogen interaction

The CCL-6 cell monolayer was grown on 24x24mm sterile cover slips on the bottom of a 6-well culture plate and incubated in RPMI medium as previously reported for 24h. A fungal inoculum of 100µl of 1x10⁸ propagules/ml solution was added to the culture plate and incubated for 6h at 37°C in a 5% CO₂ atmosphere. The test solutions were added to the inoculums. Subsequent to treatment with *P. brasiliensis*, the cover slips containing the CCL-6 cell monolayer were washed 3 times in RPMI medium to remove the non-adhered fungal cells and fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer for 2h. The adhesions events for each treatment were evaluated in triplicate by light microscopy using Nomarsky optics. Fifty randomly chosen microscopic fields were assessed, and the adhesion events were counted. The adhesion inhibition rates were estimated by the following equation: $IR = 100 - [(AdCarb \times 100)/AdCont]$, where: IR = inhibition rate; AdCarb = number of fungal adhered cells on carbohydrates treated cells; and AdCont = number of fungal adhered cells on the control

Adhesion inhibition assay

The ability of carbohydrates to interfere with the cellular interaction between *P. brasiliensis* and CCL-6 cell was assessed. The carbohydrates D-mannose, N-acetyl-glucosamine, D-fucose, sorbitol (Calbiochem Co. - San Jose CA, USA) at concentrations of 1, 25, 50 and 75mM, and D-glucosamine and D-galactosamine (5, 10 and 25mM) or fructose (75mM) (Sigma-Aldrich, St. Louis MO, USA) were added to the fungal inoculums prior to the infections. No carbohydrates were added to the control group.

Immunolocalization

To better characterize the adhesion of *P. brasiliensis* to host cells, 6h post-infection, the cover slips containing the CCL-6 cell monolayer infected with the fungus were treated with a rabbit extract total anti-*P. brasiliensis* antibody (produced at the Cell Immunology Laboratory at UFMG-Brazil and generously provided by Dr. Goes de Miranda) diluted 1:100 in PBS (0.1M) containing 5% BSA for 2h at room temperature. Following incubation, the cover slips were treated with rabbit anti-IgG monoclonal antibody conjugated to FITC (Sigma-Aldrich, St. Louis MO-USA) diluted 1:20 in PBS containing 5% BSA for 2h. The cover slips were examined for fluorescence (Olympus IX70 - BW filter). When necessary, the images obtained from the microscope were acquired using an Optomitricks camera (DMI - 470) and processed using the Image Pro-plus software (Media Cybernetics - London - England).

Labeling of the leveduriform cell with fluorescein-labeled lectin

Leveduriform cells of *P. brasiliensis* were incubated in presence of FITC-labeled lectins [wheat germ agglutinin (WGA), concanavalin A (ConA) *Arachis hypogea* (PNA) and *Glycine max* (SBA) - (EY Laboratories, Inc - California (USA))] diluted in RPMI (sigma) supplemented with 10% FBS and containing 0.0005% Mg²⁺ and Ca²⁺ at concentration of 100µg/ml for 1h at room temperature. The cells were washed three times with RPMI and observed by fluorescence microscopy (Olympus BX-41 - WB filter). When necessary, the microphotographs were recorded using a DMI-470 video camera and the Image Pro-plus software.

Statistical analysis

The number of cell adhesions per field of the treated and untreated cells were compared using the paired *Student's t test* with a significance level of $p < 0.05$ and converted into a percent of adhesion inhibition. The statistic analysis was performed using BioEstat ver.5.0 (2007).

RESULTS

Host cell/parasite interaction

The *P. brasiliensis* cells that adhered to the CCL-6 monolayer were morphologically different from the non-adhered fungal cells (**Figure 1**). The non-adherent cells had a traditional shape of a *pilot wheel* (**Figure 1A**), and the propagules normally had multiple buds and pseudo-hyphae were rarely observed. However, the adhered cells had an abundant formation of pseudo-hyphae (**Figure 1B**). These structures were longer than the usual pseudo-hyphae observed on *P. brasiliensis* cells growing on solid medium. On the host cells, the propagules were firmly attached and were not removed, even during strong agitation. After adhesion, the *P. brasiliensis* mother cells emitted several expansions that grew on the top of the epithelial cell monolayer. Normally, pseudo-hyphae are observed on the surface of the monolayer, but commonly pseudo-hyphae were observed growing under the surface (**Figure 1B**; arrows). When the infected monolayer was treated with fluorescent antibodies, the fungal structures located above the surface were fluorescently stained, while the pseudo-hyphae growing under the surface were not (**Figure 1C**). The monolayer that was not supplemented with carbohydrates had adhesion rates ranging from 75 to 300 events per observation field.

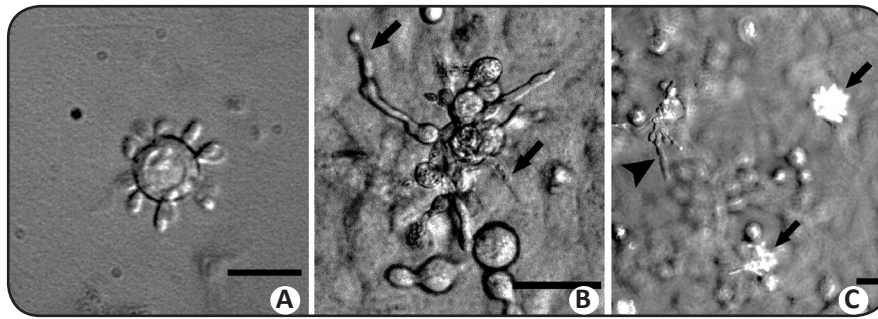


FIGURE 1 - Adhesion and invasion of *Paracoccidioides brasiliensis* cells on a CCL-6 cell layer. A: Nomarsky microphotograph of non-adhered *Paracoccidioides brasiliensis* cells in a traditional pilot wheel shape. B: Nomarsky photomicrograph of a *Paracoccidioides brasiliensis* propagule with intense pseudo-hyphal formation. Some of the pseudo-hyphae are growing underneath the CCL-6 layer (arrow). C: Cell layer treated with fluorescent anti-*Paracoccidioides brasiliensis* antibody. Arrowhead depicts fungal pseudo-hyphae growing underneath the cell layer; Arrows indicate fluorescent fungal cells on the top of the monolayer. Bar=10µm.

Adherence inhibition

The addition of D-mannose, D-fucose and N-acetyl-glucosamine at concentrations of 25, 50 and 75mM in the interaction medium significantly reduced the adhesion of *P. brasiliensis* to the CCL-6 cells. The rates of adhesion inhibition in the treatments with D-mannose were reduced to 60, 78 and 70% at concentrations of 25, 50 and 75mM, respectively. The decrease in adhesion with the addition of D-fucose and N-acetyl-glucosamine was 60, 79 and 63 and 58, 81 and 82% at the same concentrations, respectively. Practically no effect was observed after treatment with Sorbitol and Fructose. Were observed significant reduction in all concentrations and tested substances ($p < 0.05$) in relation with the control except the to sorbitol at 25mM (Figure 2). At concentrations of 1mM, no effect was observed, and at concentrations higher than 75mM, the monolayer cell death was higher than 80%. At 12h after the addition of carbohydrates, the adhesion rates were not significantly different from the untreated controls. To assess whether the decrease of adhesion inhibition was due to the sequestration or metabolization of soluble carbohydrates, mannose, N-acetyl-glucosamine and the non-metabolized carbohydrate fructose were freshly added at 8h after the initial carbohydrate treatment. Similar levels of adhesion inhibition were observed after the additional carbohydrate treatment as compared with the 4h treated cells (Figure 3).

The carbohydrates D-glucosamine and D-galactosamine were added at concentrations of 5, 10 and 25mM because higher

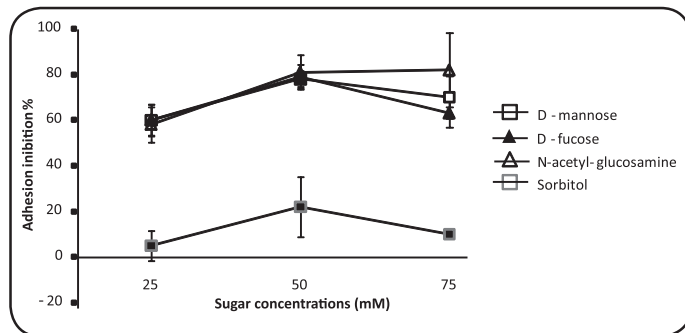


FIGURE 2 - Percentage adhesion inhibition of *Paracoccidioides brasiliensis* cells to CCL-6 monolayer at 6 h after treatment with Sorbitol (■), D-mannose (□), D-fucose (▲), and N-acetyl-glucosamine (△) at concentrations ranging from 25 to 75 mM.

concentrations were detrimental to the host cell. The adhesion reductions were estimated at 0, 30, 37% for D-glucosamine and 29, 40 and 37% for D-galactosamine for the corresponding concentrations, respectively. The results were significant only for tests with 5 and 25 mM (Figure 4).

Lectin labeling

The ConA-FITC and WGA-FITC conjugates effectively labeled *P. brasiliensis* yeast cells (Figure 5). Most importantly, the labeled cells did not fluoresce if treated with 100mM of D-mannose (ConA-FITC pre-treated propagules) and N-acetyl-glucosamine (WGA-FITC pre-treated propagules) (data not shown). We were unable to detect PNA-FITC and SBA-FITC labeling, even after enhancing the camera detection by frame averaging and increasing the camera sensitivity. The adhered *P. brasiliensis* cells were also labeled with the ConA-FITC and WGA-FITC conjugates (Figures 5A and 5B). A small amount of fluorescence the CCL-6 mammalian cell was also observed (Figure 5C).

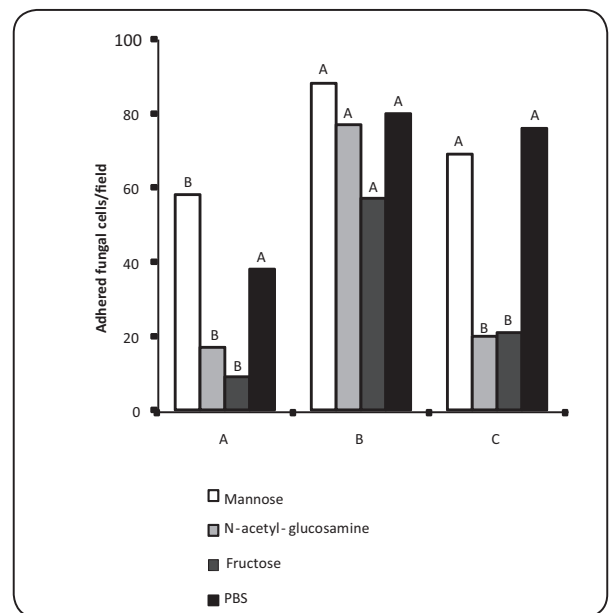


FIGURE 3 - Number of *Paracoccidioides brasiliensis* cells adhered to a CCL-6 monolayer after treatment with 75 mM mannose (□), N-acetyl-glucosamine (▨), fructose (■) or PBS buffer (■) after 4 (A), and 12 (B)h of sugar addition or after a second addition of the sugar 8h after incubation (C).

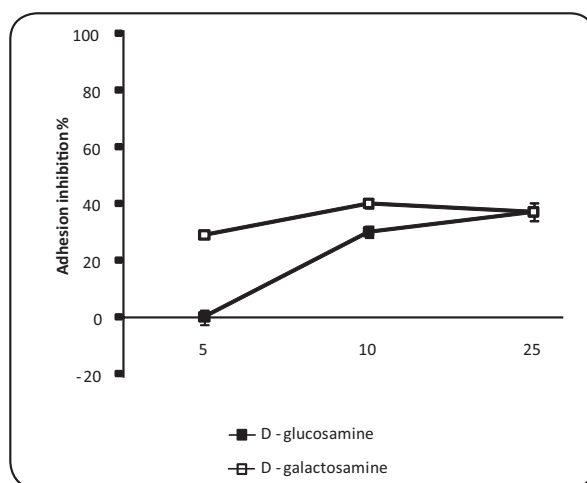


FIGURE 4 - Percentage of adhesion inhibition of *Paracoccidioides brasiliensis* to the CCL-6 monolayer at 6h after treatment with D-glucosamine (■) and D-galactosamine (□) at concentrations ranging from 5 to 25mM.

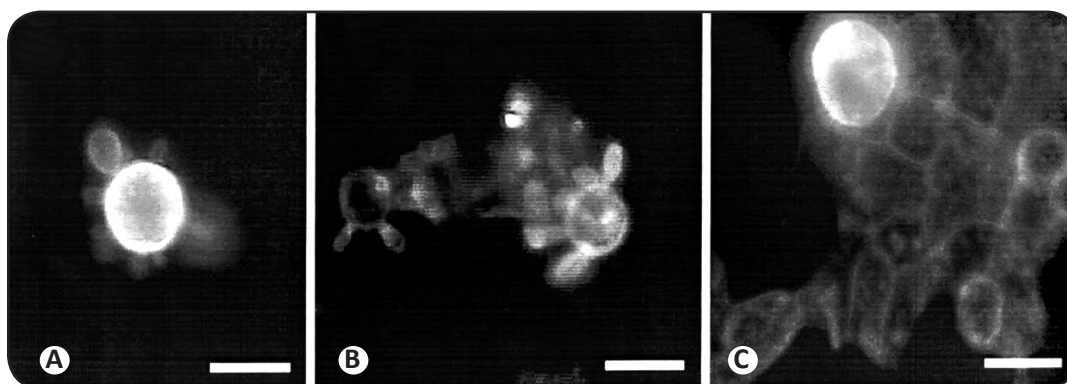


FIGURE 5 - *Paracoccidioides brasiliensis* cells after treatment with fluorescent lectins. A: Pilot wheel *Paracoccidioides brasiliensis* cells stained by WGA-FITC conjugate. Note the intense fluorescence of the mother cell. B: Fungal cells labeled by ConA-FITC conjugate. The cells were evenly labeled. C: *Paracoccidioides brasiliensis* cell adhered to CCL-6 cells after treatment with the WGA-FITC conjugate. Note the intense labeling of the fungal cell and the weak signal in the CCL-6 cell.

WGA: wheat germ agglutinin; Con-A: *Concanavalia eosiformes* agglutinin; FITC: fluorescein isothiocyanate.

DISCUSSION

Most of our knowledge concerning the interaction between *Paracoccidioides brasiliensis* and its host cell is derived from studies using *in vitro* cell culture models^{6,7,14}. However, although insightful, these reports did not assess the role of particular components in the infection. Little is known about adhesion mediators produced by *P. brasiliensis* during the course of the infection. The gp₄₃' is the only candidate molecule presumed to play a role in the regulation of the fungal propagule to its host⁸. The effect of gp₄₃ in adhesion has been inferred from indirect evidence due to the difficulty of providing direct proof. It has been previously shown that the purified glycoprotein is able to bind to laminin *in vitro*, and *P. brasiliensis* cells pre-treated with laminin bind more effectively to Madin-Darby canine kidney cells. Furthermore, when *P. brasiliensis* laminin-treated cells were injected into guinea pigs, a higher number of granuloma was formed in the host in comparison with the guinea pig injected with the untreated

fungal cells⁸. Although the adhesion function of gp₄₃ adhesion function was not directly tested. The gp₄₃ is rich in mannose and mannose interfered in the adhesion of *P. brasiliensis* to CCL-6 cell. The addition of monosaccharide D-mannose to the interaction medium prior to infection significantly reduces the adhesion of *P. brasiliensis* to CCL-6 cells. The adhesion reduction was more significant at a concentration of 50mM, reaching 79% inhibition (Figure 2). The participation of mannose residues in the interaction mechanism between the host and pathogen was also observed in other infection models^{12,20}.

Here, we report that some carbohydrates were able to diminish adhesion *in vitro* at rates as high as 90% and therefore, it's the role of these carbohydrates in the natural adhesion process has to be investigated. The role of carbohydrates in modulating fungus/host cell adhesion has been reported previously^{9,21}.

The addition of monosaccharide D-mannose to the interaction medium prior to infection significantly reduces the adhesion of *P. brasiliensis* to CCL-6 cells. The adhesion reduction was more

significant at a concentration of 50mM, reaching 79% inhibition (**Figure 2**). The participation of mannose residues in the interaction mechanism between the host and pathogen was also observed in other infection models^{13,20}. Most of the work, however, was conducted using manopyranosides, lectins or glycosylated substrates as adhesion inhibitors. Normally, sugars linked to carrier protein are used to prevent the carbohydrate from being metabolized or sequestered by the cells during the assay. In our case the sugar itself has an effect on adhesion. Interestingly, if the co-cultivation was performed for a longer time, the adhesion inhibition would decrease but could be restored by the addition of mannose to the cultivation media. The restoration is probably due to the fact that the cell effectively processes mannose over time and therefore, its concentration decreases in the incubation medium, resulting in the decrease of its effect on inhibition of adhesion. An increase in mannose concentration restores the inhibition. Similar results were observed with N-acetyl-glucosamine (**Figure 3**). Fructose is a sugar that is not metabolized by either the fungal or the host cells and did not have an effect on adhesion.

Another competitor of adhesion utilized in our studies was D-fucose. Fucose is a deoxyhexose that is present in a wide variety of organisms. In mammals, fucose-containing glycans have important roles in blood transfusion reactions, selectin-mediated leukocyte-endothelial adhesion, host-microbe interactions, and numerous ontogenic events, including signaling by the Notch receptor protein family²¹. The involvement of fucose in the interaction between fungi and its host was established by the characterization of the fucose receptor in the germination tubes of *C. albicans*²².

The addition of N-acetyl-glucosamine to the interaction medium significantly decreases the adhesion percentage of the fungi to the cellular host type. The adhesion reduction was more significant at concentrations of 50 and 75mM (**Figure 2**). Results similar for *Fonsecaea pedrosoi* pre-incubated with the N-acetyl-glucosamine-BSA conjugate¹³.

D-glucosamine and D-galactosamine were also able to decrease the adhesion index of *P. brasiliensis* to CCL-6 cells (**Figure 4**). Those sugars have been implicated in the adhesion of *Entamoeba histolytica* to epithelial cells and *C. albicans* to lymphocytes^{10,23}.

Carbohydrates have been described as important adhesion mediators in many biological models, and this idea remains consistent with regard to the interaction of *P. brasiliensis* and epithelial CCL-6 cells. The cell treatment with those carbohydrates actually decreases the adhesion efficiency of the fungal cells. Unfortunately, even though and inhibitory effect was observed, we were not able to directly implicate the carbohydrates in the physiological event of adhesion. Several other molecules could also play a role in the process and would be not detected by our experimental approach.

Therefore, further studies are necessary to identify and characterize the molecules with affinity for carbohydrates that are present on the fungal surface and/or its host. Lectins are described as proteins that able to bind particular sugars. In several other biological systems, lectins are believed to be the cellular receptor for carbohydrates that modulate adhesion and cell recognition, for example. To assess whether the lectins are capable of recognizing carbohydrates on the surface of *P. brasiliensis*, we labeled yeast cells with ConA, WGA, PNA and SBA lectins conjugated to FITC. Of the four lectins assessed, only ConA and WGA labeled *P. brasiliensis* cells.

The Con-A lectin specifically recognized α -mannose and α -glucose, while WGA bound to N-acetyl-glucosamine. These sugars were also able to inhibit *P. brasiliensis* adhesion to CCL-6 cells; therefore, one might suggest that those sugars are present on the *P. brasiliensis* surface and are the receptors responsible for cell-to-cell adhesion. However, PNA, which binds terminal galactose, and SBA, which recognizes N-acetyl-galactosamine and galactose, were not able to detect these sugars on the surface of *P. brasiliensis* cells. Inhibitory assays with N-acetyl-galactosamine showed a strong decrease of adhesion therefore, one would expect that the SBA lectin would recognize such a sugar on *P. brasiliensis* cells. One possible argument for the apparent contradiction in the data is that the sugar would be present on the host cell surface.

However, SBA-FITC was also unable to show binding sites on the CCL-6 cells (data not shown). The role of galactosamine is therefore unclear. Adhesion is an early event in most of the pathogen/host interactions and is crucial for efficient disease development. Several mediators are constantly being implicated as having a role in the adhesion event, but a definitive experiment is yet to be proposed. Our results, are circumstantial, and only emphasize the effect of *P. brasiliensis* surface carbohydrates in the adhesion/recognition process. An enhanced knowledge of the mediators of adhesion on the biotrophic fungus *P. brasiliensis* will be useful in the future for the development of more efficient and less harmful methods for disease treatment and control.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

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ABSTRACT IN PORTUGUESE

A influência de carboidratos na interação entre *Paracoccidioides brasiliensis* e células CCL-6 (in vitro)

Introdução: Pouco se conhece a respeito dos eventos iniciais que mediam as interações entre *Paracoccidioides brasiliensis* e seus hospedeiros. Com a intenção de compreender a importância de carboidratos junto a estas interações, foram analisados os efeitos de soluções de carboidratos sobre a adesão de células leveduriformes de *P. brasiliensis* sobre culturas de células CCL-6. **Métodos:** As células fúngicas foram cultivadas com as células epiteliais e diferentes concentrações de D-fucose, N-acetyl-glucosamina, D-manose, D-glicosamina, D-galactosamina, sorbitol e frutose foram adicionadas ao cultivo no início da interação. Após 6h de tratamento, as células foram fixadas e observadas em microscópio óptico. **Resultados:** Os tratamentos utilizando D-fucose, N-acetil-glicosamina, D-manose, D-glicosamina e D-galactosamina reduziram os números de adesões quando comparados com o controle. Os tratamentos realizados com o uso de sorbitol e frutose apresentaram os mesmos resultados observados no controle. Para detectar a presença de carboidratos na superfície do fungo, propágulos de *P. brasiliensis* foram tratados com lectinas fluorescentes. WGA-FITC e Con-A-FITC se ligaram às células de *P. brasiliensis* ao contrário de SBA e PNA. **Conclusões:** O percentual de adesão entre *P. brasiliensis* e células CCL-6 foi reduzido com o uso de D-manose, N-acetil-glicosamina e D-glicosamina. O uso de lectinas marcadas sugeriu a presença de N-acetil-glicosamina, α -manose e α -glicose na superfície de *P. brasiliensis*. Estes resultados contribuem para o aumento do conhecimento relacionado aos mediadores de adesão de *P. brasiliensis*, e poderão ser utilizados no futuro para o desenvolvimento de medidas mais eficientes para o controle e tratamento deste patógeno.

Palavras-chaves: Paracoccidioidomicose. Adesão. Células CCL-6. Lectina fluorescente.

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