LYSOZYME PLAYS A DUAL ROLE AGAINST THE DIMORPHIC FUNGUS *Paracoccidioides brasiliensis*

Damaris LOPERA(1), Beatriz H. ARISTIZABAL(1,2), Angela RESTREPO(1), Luz Elena CANO(1,3) & Ángel GONZÁLEZ(1,3)

SUMMARY

In order to determine the role of lysozyme, an antimicrobial peptide belonging to the innate immune system, against the dimorphic fungus *Paracoccidioides brasiliensis*, co-cultures of the MH-S murine alveolar macrophages cell line with *P. brasiliensis* conidia were done; assays to evaluate the effect of physiological and inflammatory concentrations of lysozyme directly on the fungus life cycle were also undertaken. We observed that TNF-α-activated macrophages significantly inhibited the conidia to yeast transition ($p = 0.0043$) and exerted an important fungicidal effect ($p = 0.0044$), killing 27% more fungal propagules in comparison with controls. Nonetheless, after adding a selective inhibitor of lysozyme, the fungicidal effect was reverted. When *P. brasiliensis* propagules were exposed directly to different concentrations of lysozyme, a dual effect was observed. Physiologic concentrations of the enzyme facilitated the conidia-to-yeast transition process ($p < 0.05$). On the contrary, inflammatory concentrations impaired the normal temperature-dependant fungal transition ($p < 0.0001$). When yeast cells were exposed to lysozyme, irrespective of concentration, the multiple-budding ability was badly impaired ($p < 0.0001$). In addition, ultra-structural changes such as subcellular degradation, fusion of lipid vacuoles, lamellar structures and interruption of the fibrilar layer were observed in lysozyme exposed conidia. These results suggest that lysozyme appears to exert a dual role as part of the anti-*P. brasiliensis* defense mechanisms.

KEYWORDS: Lysozyme; TNF-α; Alveolar macrophages; *Paracoccidioides brasiliensis*.

INTRODUCTION

In respiratory tract secretions, lysozyme is the most abundant antimicrobial peptide, and its local levels increase tenfold during inflammation[31]. The bactericidal properties of lysozyme are primarily ascribed to its enzymic activity, resulting in hydrolysis of β-linkage between the N-acetylglucosamine and N-acetylmuramic of the bacterial peptidoglycan causing cell lysis. However, an increasing body of evidence supports the existence of non-enzymatic and/or non-lytic modes of action still not well characterized[11,20]. The antimicrobial action of lysozyme contributes to the innate host defense and acts in concert with the mechanical and phagocytic clearance mechanisms of the host interacting also with other cationic proteins[7]. Lysozyme is present in the phagocytic cells' granules of neutrophils, as well as in macrophages, monocytes and epithelial cells. After its discovery by Alexander Fleming in 1922, the enzymic action of lysozyme has been extensively characterized; however, its role in the host-parasite interaction has been studied more intensely in bacteria than in fungi.

Although lysozyme is constitutively expressed, providing an ever present defense, pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), dramatically increase lysozyme levels. TNF-α is a pleiotropic cytokine capable of increasing the respiratory burst stimulating lysozyme overproduction by human macrophages[25,30].

Paracoccidioidomycosis (PCM) is one of the most important endemic mycosis in Latin America. This mycosis affects primarily the lung and is frequently followed by dissemination to mucosa, skin, adrenals and other organs and systems[2,27]. The natural infection is acquired by inhalation of *Paracoccidioides brasiliensis* conidia, propagules that convert into the invasive yeast form once in the lungs[27].

In a murine model of PCM, it was observed that resistant mice (AS/n) infected with *P. brasiliensis* yeast cells were able to control the infection when compared with susceptible mice (B10A); additionally, high levels of TNF-α were found in the former indicating an important role of this cytokine in host's defense[22].

It is also known that the mononuclear phagocytic system plays a fundamental role in resistance to *P. brasiliensis*[17]. On the same token, macrophages activated with interferon gamma (IFN-γ) or TNF-α have antifungal properties against *P. brasiliensis* that can be dependent or independent of nitric oxide production, respectively[12,13]. Although TNF-α is clearly required, the mechanism by which this cytokine contributes

(1)Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia.
(2)Molecular Diagnosis Laboratory, Hospital Pablo Tobón Uribe, Medellín, Colombia.
(3)Microbiology School, Universidad de Antioquia, Medellín, Colombia.
Correspondence to: Ángel González, Corporación para Investigaciones Biológicas, CIB, Carrera 72A No. 78B-141, Medellín, Colombia. Phone: (574) 441-0855, ext. 219, 221. Fax: (574) 441-5514. E-mail: angelgo39@hotmail.com; agonzalezm@cib.org.co.

to fungus control is not fully understood. In this study, we attempted to determine if lysozyme participates in the fungicidal/fungistatic mechanism against *P. brasiliensis* conidia or if this enzyme would affect either the conidia-to-yeast transition or the multi-budding process of this pathogenic fungus.

MATERIALS AND METHODS

Reagents: Hen egg white lysozyme, immunochemicals and all reagents employed in this study were obtained from Sigma Chemical Co. (St Louis, USA), unless specified.

Production and collection of *P. brasiliensis* conidia: *P. brasiliensis* strain deposited in the American Type Culture Collection (ATCC No. 60855) and originally isolated from a Colombian patient was used throughout this study[28].

The fungus was maintained at 18 ºC in its mycelial form by successive transfers on synthetic McVeigh-Morton (SMV) medium[26]. Growth was then transferred to an Erlenmeyer with liquid SMV and incubated for 10-15 days (18 ºC) with constant shaking at 150 rpm (Model G-2 gyratory shaker, New Brunswick Scientific, Co. New Brunswick, N.J.). After this time, the growth was collected, homogenized in a blender (Eberbach container assembly semi-micro press with fit cover) for 15-20 seconds in four intervals of four seconds each, and plated in Petri dishes containing a media that stimulates sporulation, namely, water agar medium and dextrose salts agar[3,28]. The culture dishes were incubated at 18 ºC for periods of 3-5 months and inspected periodically for the presence of contaminating bacteria or mold growth.

Culture dishes were washed with 0.85% saline solution plus 0.01% of Tween-20; this suspension was then shaken at 250 rpm for 45 min at 18 ºC in Erlenmeyer flask containing glass beads. The homogenized suspension was sonicated twice for 15 seconds (7Hz) at 4 ºC with one minute intervals (Sonicator model 200, Branson Ultrasonic Co, Danbury,CT)

The conidia were separated from the mycelium fragments through Percoll gradients[16] and finally, their viability was determined using the fluorescein diacetate-ethidium bromide fluorescence method[4].

All procedures leading with conidia were carried out with extreme precautions and were manipulated in biological safety cabinets class IIA.

Production and collection of *P. brasiliensis* yeast cells: Yeast cells cultures were maintained by periodic transfers each four days to Sabouraud dextrose medium, supplemented with 0.2% asparagine (Sigma) and 0.01% thiamine (Sigma) at 36 ºC. The growth was washed with 2 mL of phosphate buffer and adjusted to 25 mL of this buffer. In order to separate the yeast's buds from the mother cell (≤ 2 buds); the suspension was sonicated twice for 20 seconds (7Hz) at 4 ºC with one minute intervals. After separation, the yeasts were counted in a hemocytometer. The sonication procedure did not alter the yeast's viability (standardized procedure in our laboratory).

Alveolar Macrophages: The MH-S murine alveolar macrophages cell line, originally collected from BALB/c mice and deposited in the European Collection of Cell Cultures (EACC, No. 95090612), were cultured at 37 ºC, 5% CO_2 in RPMI-1640 medium supplemented with 15% of foetal bovine serum, 2 mM glutamine and 0.05 mM 2-β-mercaptoethanol[21].

Monolayer treatments: Confluent MH-S cell monolayers were harvested, counted in a hemocytometer and their viability determined by trypan blue exclusion. Cellular suspension was adjusted at 6.25 $\times 10^4$ viable cells/mL and 0.4 mL of this suspension was deposited in each eight-well LabTek[MR] chambers slides (Cat number 177402; Nalgene Nunc International, Naperville, IL).

Macrophages were treated with 10 ng/mL of TNF-α (Cat number T-7539, Sigma) 24 h before infection[12] and/or with a competitive inhibitor of the enzymatic activity of lysozyme, N, N', N"-triacetylglucosamine (NAG3), also referred as N, N', N"-triacetylchitotriose (Cat number T-2144 St, Sigma)[1,19]. In order to inhibit the constitutively lysozyme production, the inhibitor was added simultaneously with TNF-α before infection. The final concentration of this trisaccharide was 140 μM, which represents four molecules of the inhibitor per each one of lysozyme. In addition, non-infected and non-treated macrophages were also studied and used as controls.

Infection of macrophages: Conidia were suspended in RPMI containing 30% (vol/vol) fresh mouse serum from BALB/c mice. Conidial suspensions were incubated at 37 ºC for 20 min for opsonization to take place[5]. Monolayer macrophages were inoculated with 0.02 mL of the conidial suspension, which gave a conidium-to-macrophage ratio of 1:5. The chambers were incubated at 37 ºC, 5% CO_2 for 96 h.

Determination of the conidia -to-yeast transition process: Slides removed from this chambers were fixed with cold absolute methanol, air dried, and stained with silver methenamine (Grocot's stain) to determine the presence of intracellular fungal propagules[12]. The monolayers were observed with light microscopy (40X) and the number of intracellular fungal propagules was counted, distinguishing between conidia (non-transformed) and yeast cells (transformed). More than 200 intracellular propagules were counted and the percent of transformation was calculated as number of intracellular yeast cells/ 200 intracellular fungal cells × 100.

Colony forming units (CFU): Other chamber wells were used to evaluate the CFU's number. The monolayers were lysed with 200 μL of distilled water for 30 min at 37 ºC and the suspension was harvested and adjusted to a final volume of 1 mL; 350 μL of the latter suspension were plated in agar plates (60 per 15 mm of diameter) with BHI plus 5% of glucose (Cat Number G5400, Sigma), 300 μM EDTA and 4% horse serum (Gibco, Grand Island, NY, USA; Cat Number 16055-122)[18]. The inoculated agar dishes were incubated at 36 ºC for 15 days, and colonies per plate were counted after eight to 10 days of incubation.

Fungal cultures in presence of lysozyme: A stock solution of lysozyme was dissolved in sterile distilled water at a concentration of 16 mg mL^{-1} and sterilized by passage through a 0.22 μm Millipore filter MFS 25 (Millipore Co., Bedford, MA, USA).

Conidial suspension at a final concentration of 50,000 conidia per

mL were transferred to each of six tubes containing 1.0 mL of filtered BHI supplemented with 1% glucose (Cat Number G5400, Sigma), 0.01% thiamine and 0.02% L-cystine (Cat number C-8755, Sigma). Each tube received a different lysozyme volume, reaching final concentrations of 0, 50, 100 µg/mL (physiological concentrations) and 200, 400 and 800 µg/mL (inflammatory concentrations)[24]. Aliquots of 200 µL were plated by triplicate in 96 wells microplates (Nunclon Surface, Nunc Brand) and incubated at 36 °C ± 0.5 °C for 96 hours.

Dimorphic transition and budding changes determination: To determine if lysozyme induced morphological changes in *P. brasiliensis* propagules, we evaluated the capacity of the conidia to convert into yeast, and also yeast budding.

After 24, 48, 72, and 96h of incubation, the kinetics of conidium-to-yeast transition was determined. Morphological evaluation was made in an inverted microscope (Lietz Diavert 903, Germany). The number of single or multi-budding yeast cells was counted in each well to evaluate the transition process.

In order to evaluate lysozyme effects on the budding process, yeast cells obtained after the sonication process were exposed to the same concentrations of lysozyme and plated to a final concentration of 1 x 10^3 yeasts per well. After 24, 48, and 72 hours post-incubation the number of multi-budding yeast cells were counted.

Ultrastructural changes determined by transmission electron microscopy: Conidia treated with lysozyme at concentrations of 50 µg/mL and 800 µg/mL were harvested by centrifugation after 96h of incubation, washed once in phosphate buffer and fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) overnight. Samples were processed according to standard protocols[9]. In brief, the fixed samples were treated with 2% OsO4 at room temperature for one hour, dehydrated slowly with ethanol and embedded in Epon resin. Thin-sections were observed in a Zeiss EM electron microscope.

Statistical analyses: Results are expressed as the mean ± standard deviation for at least three experiments by duplicate (n = 6). Comparisons between groups were analyzed by the Student *t* test using the GraphPad Prism program, version 4.0 (California, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Intracellular transition of *P. brasiliensis* conidia after lysozyme inhibition: As shown in Fig. 1A and 1B, 96h after infection of the macrophages with *P. brasiliensis* conidia, fungal propagules appeared internalized by phagocytes. In non-activated and non-lysozyme treated macrophages (control group), we observed that approximately 50% of these propagules had undergone intracellular transition. The addition of TNF-α resulted in significant ($p = 0.0043$) inhibition of the conidium-to-yeast transition process in comparison with the control group (Fig. 1C). Nonetheless, this effect was reverted in the presence of the lysozyme inhibitor ($p = 0.0022$), a reagent that also inhibited the transition process in the non-activated macrophages exposed to the same inhibitor, in a way similar to that TNF-α.

Lysozyme participation in the fungicidal/fungistatic effect

exerted by TNF-α-activated macrophages infected with *P. brasiliensis* conidia: The ability of the alveolar macrophages to destroy fungal propagules was evaluated by determining CFU's number. As shown in Fig. 1D, only macrophages activated with TNF-α exerted a
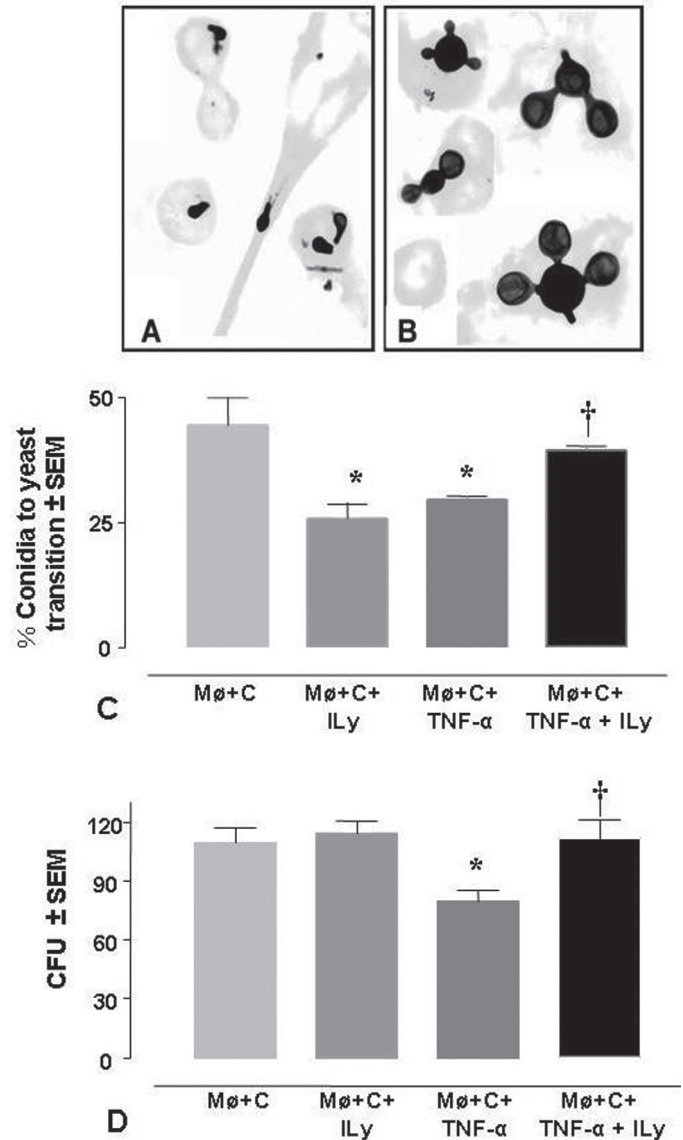

Fig. 1 - *P. brasiliensis* intracellular transition and CFU of conidia after lysozyme inhibition in normal or TNF-α activated Møs. A) Morphology of intracellular *P. brasiliensis* conidia, and B) yeast cells observed after 96 h of co-culture with alveolar macrophages (Magnifications 1000X). C) Intracellular transition of *P. brasiliensis* conidia, after lysozyme inhibition in activated or non-activated macrophages with TNF-α for 96 h at 37 ºC (n = 6). Bars show the mean ± SEM. *p < 0.01 between the control group (non-activated Møs infected with *P.brasiliensis* conidia) and the group of TNF-α-activated Møs. † *p* < 0.01 between TNF-α-activated Møs and Møs with the same treatment plus lysozyme inhibitor. D) Colony forming units (CFUs) recovered from alveolar Møs activated or non-activated with TNF-α, infected with *P. brasiliensis* conidia and treated with lysozyme inhibitor after incubation for 96 h at 37 ºC (n = 18). The bars show the mean ± SEM. * *p = 0.0044* between TNF-α-activated Møs and control group (Mø+C). † *p = 0.0054* between TNF-α-activated Møs and Møs with the same treatment plus lysozyme inhibitor.

fungicidal effect on *P. brasiliensis* conidia ($p = 0.0044$). This group of phagocytes destroyed 27% more propagules than the non-activated macrophages, an effect that was reverted in the presence of the lysozyme inhibitor ($p = 0.0054$).

Effect of lysozyme on the fungal transition: Fig. 2A and 2B show the effect of different concentrations of lysozyme in the transition of *P. brasiliensis* conidia, and the capacity of this new yeast cells to bud. The susceptibility of conidia was dependent on lysozyme concentration. Physiological concentrations of the enzyme (50 and 100 µg/mL) (Fig. 2A) facilitated the conidia-to-yeast transition process, $p < 0.001$ and $p < 0.05$, respectively. On the contrary, inflammatory concentrations of lysozyme (400 and 800 µg/mL) impaired fungal transition ($p < 0.001$), as well as its budding ability ($p < 0.001$) (Fig. 2A and 2B).

Effect of lysozyme on the ability of yeast to bud: In order to confirm the effect of lysozyme on the multi-budding capacity described above, the effect of lysozyme directly on the tissue yeast form was evaluated. As observed in Fig. 2C, yeasts incubated for 72 hours in the presence of different lysozyme concentrations presented a smaller number of blastoconidia in all groups exposed to this enzyme, in contrast to control yeast (no lysozyme) which exhibited multiple budding. This inhibition became apparent from the first 24h when only 25% of the yeasts exposed to the different concentrations of lysozyme were able to begin their budding, in contrast with 45% observed in the control yeast. Similar findings were observed after 48 and 72h post-incubation being more evident in the latter. After 72h post-incubation multiple-budding increased to 72% in the control group in contrast to yeasts exposed to lysozyme which rose only less than 50%. Concentrations of lysozyme were not statistically significant different from each other.

Ultrastructural changes: Transmission electron micrograph of untreated *P. brasiliensis* conidia is presented in Fig. 3A. Conidia show their characteristic oval or pear shape, homogenous intracytoplasmic lipid bodies and well delimited cell wall. These propagules had a clear thin layer near the plasma membrane that delimited the periplasmic space. The cellular wall was surrounded by a fibrillar material that, in some cases, appeared discontinuous in the wall; none of untreated *P. brasiliensis* conidia studied shown an apparently change on their ultrastructure.

Nonetheless, propagules exposed to physiologic and inflammatory concentration of lysozyme (50 µg/mL and 800 µg/mL, respectively) showed similar changes. The most evident ultrastructural changes were observed in the cytoplasm where fusion of lipid bodies and formation of lamellar structures as a product of subcellular degradation were recorded (Fig. 3B and 3D). In some cells it was evident cytoplasm reduction (Fig. 3D). Although the interruption of fibrillar layer was also observed in control cells, it was more evident in conidia exposed to lysozyme (Fig. 3C).

DISCUSSION

These results reveal for the first time that lysozyme probably participates in the restriction and control of the pathogenic fungus *P. brasiliensis* cooperating with TNF-α-activated macrophages in their fungicidal/fungistatic mechanisms exerted against the infective propagules.

TNF-α-activated macrophages impaired both the transition process and the viability of conidia, effects that could be reverted in the presence of the lysozyme inhibitor, suggesting that this enzyme contributes to
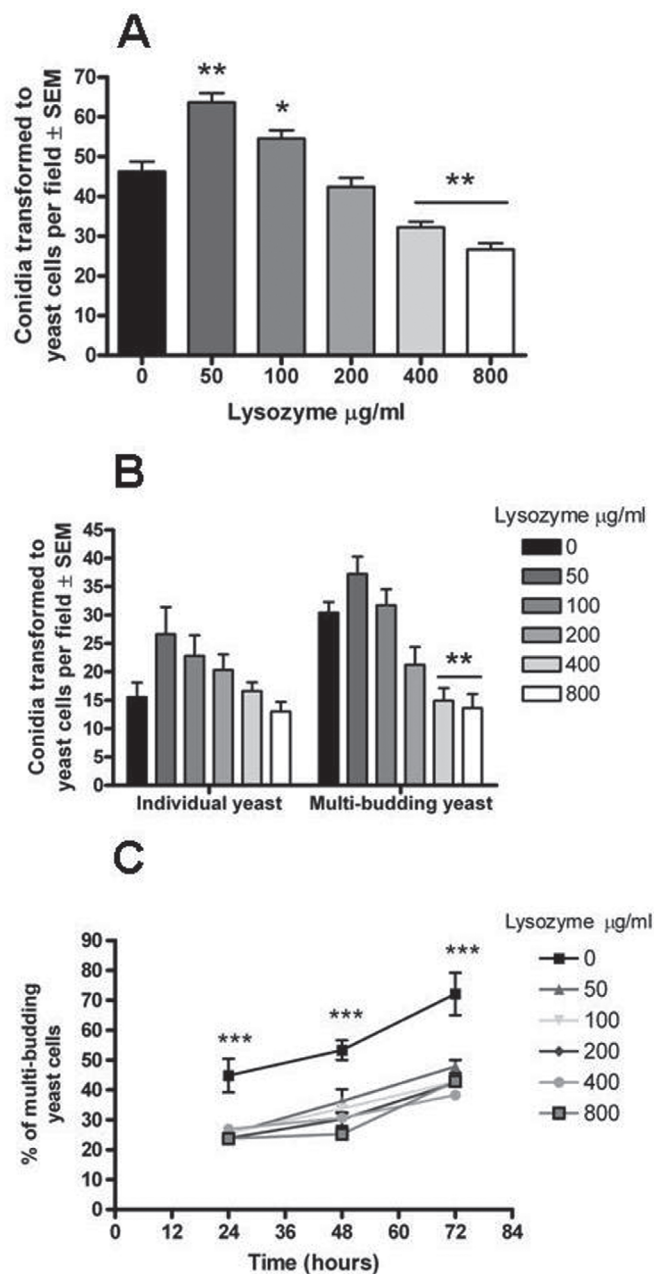

Fig. 2 - Effects of different concentrations of lysozyme on *P. brasiliensis* conidial transition process and the budding capacity of yeasts. A) Number of conidia per field that underwent dimorphic transition to yeast cells. B) Transition according to the observed propagules, individual yeasts and multiple budding yeast cells. The bars show the mean ± SEM. **$p < 0.001$ *$p < 0.05$ when compared to the control (cultures without lysozyme). C) Kinetic of *P. brasiliensis* budding after 24, 48 and 72 hours of yeast exposure to lysozyme. Bars show the mean ± SEM of percentage of multiple budding yeast of three experiments run in duplicate ($n = 6$). ***$p < 0.0001$ when compared yeast cells without lysozyme (control) and yeasts in the presence of different concentration of the enzyme.
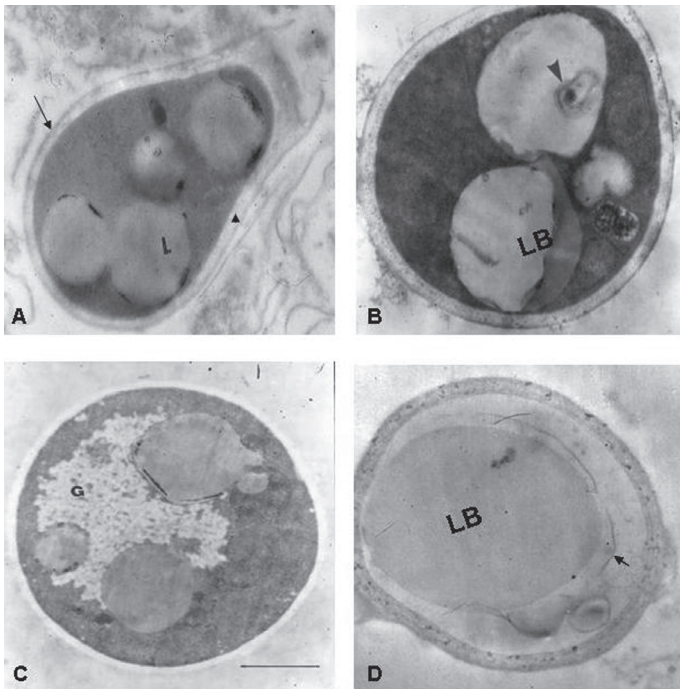
Fig. 3 - Ultrastructural changes on conidia exposed to lysozyme by transmission electron microscopy. A) Untreated conidia (control) display homogenous intracytoplasmic lipid bodies (L), arrow head indicates the periplasmic space and arrow indicates the fibrillar layer that delimits the cellular wall (Magnification 22,000X). B-D) Conidia exposed to lysozyme; B) conidia displays lamellar structure formation (arrow head) inside of a big lipid body (LB). In the cytoplasm, bodies of different sizes and densities that don't show sub-cellular definition can be observed. C) Conidia displaying normal cytoplasmic appearance with homogeneous lipid bodies and great amount of glycogen that occupies most of the cytoplasm (G). Observe that the cellular wall lacks the fibrillar layer; D) conidium exhibits a giant lipid body occupying great part of the cytoplasm (LB); in addition, non continuous membrane structures and a residual appearance in the cytoplasm can also be observed; arrow indicates protrusions of the cell wall. Bar 1.0 μm (Magnification 22,000X).

the defense mechanisms exerted by the macrophages activated with TNF-α.

We observed that when the lysozyme inhibitor was added to non-activated macrophages the transition process was impaired. Nonetheless, the viability of the fungus remained unaffected as shown by the CFU. Since we did not find a direct negative effect of the lysozyme inhibitor on *P. brasiliensis* growth (data not shown), new approaches are needed to identify the effect observed on the intracellular transition process.

TNF-α is involved in multiple steps in the antifungal and inflammatory responses against *P. brasiliensis*. This cytokine activates macrophages and is required for the sustained recruitment of inflammatory cells into granulomatous lesions[22,32]. In addition, previous studies done by our group have demonstrated that TNF-α-activated macrophages exert an antifungal effect independent of nitric oxide production[12]. According with the above results, the present study suggests that lysozyme probably contributes to the mechanisms exerted by TNF-α-activated macrophages.

In addition, in this study we observed that inflammatory concentrations of lysozyme were capable of inhibiting both conidia-to-yeast transition and budding processes.

However, a contrary effect was noticed when physiological concentration of lysozyme resulted in the increase of the transitional process. This effect should be explored further in order to understand the differential behavior according to lysozyme concentrations.

On the other hand, *in vitro* assays have shown that the budding process was inhibited by lysozyme even at concentrations lower than 50 μg/mL, suggesting that minimal amounts of this enzyme are sufficient to control the development of the fungus in its tissue form. The absence of a dose-dependent effect on budding suggests that inflammatory (higher) concentrations of this antimicrobial peptide would not be more effective against the fungus.

CANTOR *et al.*[6], using an animal model of emphysema showed that when hamsters were exposed to aerosolized lysozyme, this enzyme enhanced elastolysis, suggesting that deposition of lysozyme in the lung's extracellular matrix may enhance the progression of pulmonary emphysema. Studies performed in our laboratory have shown that in mice infected with *P. brasiliensis* conidia marked lysozyme expression is induced in the lungs during first four days post-infection (unpublished data). In addition, recently we observed marked elastolysis during this early inflammatory infiltration[14].

Additionally, in the TEM studies the most evident ultrastructural changes observed in lysozyme-treated conidia occurred in the cytoplasm where fusion of lipid bodies and lamellar structures were apparent. The mechanism responsible for these changes is not well understood and it would be difficult to attribute such effect to the sole enzymatic activity. It is known that the target of lysozyme is the polysaccharide exposed on the cell wall; however, two additional hypotheses suggested that a variety of cationic peptides (including lysozyme) might render bacteria non-viable by activating their autolytic wall enzymes leading to bacteriolysis[11]; other possibility to be considered is that lysozyme has antibacterial activities independent of its enzymatic action, probably due to its positive charge[15].

It is important to emphasize that results obtained from *in vitro* studies are not necessarily predictive of their activity *in vivo*, because this enzyme may be increased by certain cofactors such as lactoferrin, hydrogen peroxide, defensins and cathelisicidins[7,10,33]. Thus, it is possible that the effect observed *in vitro* by lysozyme could be potentiated *in vivo* by these other reactants; however, more studies to confirm the participation of these cofactors are needed.

Studies on the antifungal action of lysozyme have focused on *Candida albicans*. NISHIYAMA *et al.*[24] showed that lysozyme inhibited the separation of apparently mature *C. albicans* yeast cells from each other, and recorded also accumulation of wall-like material in the periplasmic space indicating that cell-wall components may be possible targets for this enzyme. Additionally, they found a synergistic action between lysozyme and lanoconazole, an antifungal drug.

In other study, WU *et al.*[34] showed that lysozyme had a dual action on *C. albicans*, killing the organism at higher concentrations and

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modulating the production of aspartyl proteinases, a putative virulence factor of *C. albicans*. SAMARANAYAKE *et al.*[29], reported that although generally the *Candida* species such as *C. glabrata, C. albicans, C. tropicalis* and *C. krusei*, were susceptible to lysozyme, differences in their susceptibility were noted among the different isolates within the same species. Fungi such as *Aspergillus fumigatus*, *Rhizopus orizae*[8] and *Histoplasma capsulatum*[23] also showed certain susceptibility to lysozyme, compound that mediated a fungistatic mechanism.

In this study, we observed that lysozyme induced ultrastructural and morphological changes in *P. brasiliensis* propagules; nonetheless, more studies are necessary to determine the mechanisms involved in the changes brought about by this enzyme on the fungal structures.

Finally, the knowledge gained concerning the molecules implied in the microbicidal activity generated by activated macrophages would allow identification of key molecules that may contribute to improve fungal control. Therefore, we consider that lysozyme could be a target for further developments aimed at defining its importance in the host defense against *P. brasiliensis* infection.

RESUMO

A lisozima desempenha um papel duplo contra o fungo dimórfico *Paracoccidioides brasiliensis*

Com a finalidade de determinar o papel da lisozima, um peptídeo antimicrobiano que pertence ao sistema imune inato, contra o fungo dimórfico *Paracoccidioides brasiliensis*, foram feitas co-culturas de uma linha de macrófagos alveolares murinos (MH-S) com as conídias do fungo na presença ou não do TNF-α e/ou um inibidor da lisozima; também foram feitos ensaios que avaliaram o efeito das concentrações fisiológicas e inflamatórias de lisozima diretamente sobre o ciclo de vida do fungo. Observamos que os macrófagos ativados com a citoquina tiveram um efeito significativo na inibição da transição conídia/levedura (p = 0,0043) e exerceram um efeito fungicida importante (p = 0,0044), matando mais de 27% das propágulas do fungo em comparação com os macrófagos não ativados. No entanto, após ser o inibidor seletivo da lisozima adicionado, o efeito fungicida foi revertido. Quando os propágulos do fungo foram expostos diretamente a diferentes concentrações da lisozima, um duplo efeito foi observado. Assim, as concentrações fisiológicas da enzima facilitaram o processo de transição conídia-levedura (p < 0,05). Contrariamente, as concentrações inflamatórias prejudicaram a transição fúngica (p < 0,0001). Quando as leveduras foram expostas a qualquer concentração de lisozima, sua capacidade de multi-brotação foi gravemente prejudicada (p < 0,0001). Além disso, mudanças ultra-estruturais, como a sub degradação, a fusão dos vacúolos dos lípidos, estruturas lamelares e interrupção da camada fibrilar foram observadas em conídios expostos à lisozima. Estes resultados sugerem que a lisozima poderia exercer um duplo papel no mecanismo antifúngico contra *P. brasiliensis*.

ACKNOWLEDGMENTS

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