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. The bactericidal properties of lysozyme are primarily ascribed to its enzymatic 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. 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 enzymatic action of lysozyme has been extensively characterized. 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 enzymatic 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.

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. The natural infection is acquired by inhalation of *Paracoccidioides brasiliensis* conidia, propagules that convert into the invasive yeast form once in the lungs.

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.

It is also known that the mononuclear phagocytic system plays a fundamental role in resistance to *P. brasiliensis*. 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. Although TNF-α is clearly required, the mechanism by which this cytokine contributes...
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 multinucleating process of this pathogenic fungus.

**MATERIALS AND METHODS**

**Reagents:** Hen egg white lysozyme, immunochromatographic 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.

The fungus was maintained at 18 °C in its mycelial form by successive transfers on synthetic McVeigh-Morton (SMV) medium. Growth was then transferred to an Erlenmeyer with liquid SMV and incubated for 10-15 days (18 °C) 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.

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 °C in Erlenmeyer flask containing glass beads. The homogenized suspension was sonicated twice for 15 seconds (7Hz) at 4 °C with one minute intervals (Sonicator model 200, Branson Ultrasonic Co., Danbury, CT)

The conidia were separated from the mycelium fragments through Percoll gradients 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 II A.

**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 °C. 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 °C 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 °C, 5% CO₂ in RPMI-1640 medium supplemented with 15% of foetal bovine serum, 2 mM glutamine and 0.05 mM 2-β-mercaptoethanol.

**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 x10⁶ viable cells/mL and 0.4 mL of this suspension was deposited in each eight-well LabTek chamber 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 and/or with a competitive inhibitor of the enzymatic activity of lysozyme, N, N', N''-triacetylgalactosamine (NAG3), also referred as N, N', N''-triacetethylchitotriose (Cat number T-2144 St, Sigma). 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 °C for 20 min for opsonization to take place. 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 °C, 5% CO₂ 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 (Grocott’s stain) to determine the presence of intracellular fungal propagules. 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.

**Colon formation 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 °C 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 mM EDTA and 4% horse serum (Gibco, Grand Island, NY, USA; Cat Number 16055-122). The inoculated agar dishes were incubated at 36 °C 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)\(^2\). Aliquots of 200 µL were plated by triplicate in 96 wells microplates (Nunclon Surface, Nunc Brand) and incubated at 36 ºC ± 0.5 ºC 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-\(\alpha\) 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-\(\alpha\).

**Lysozyme participation in the fungicidal/fungistatic effect exerts by TNF-\(\alpha\)-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-\(\alpha\) exerted a
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.
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., 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.

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 periplasmic space indicating that cell-wall components may be possible targets for this enzyme. Additionally, in the TEM studies the most evident ultrastructural changes observed in lysozyme-treated conidia occurred in the cytoplasm; 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; other possibility to be considered is that lysozyme has antibacterial activities independent of its enzymatic action, probably due to its positive charge.

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 cathelicidins. 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. 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. showed that lysozyme had a dual action on C. albicans, killing the organism at higher concentrations and enhancing the production of defensins and cathelicidins, leading to increased resistance to lysozyme.

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modulating the production of aspartyl proteinases, a putative virulence factor of C. albicans. SAMARANAYAKE et al.\textsuperscript{32}, 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 oryzae\textsuperscript{3} and Histoplasma capsulatum\textsuperscript{33} 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 imaturo, 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 citocitima 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 propágulos 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.

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