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INHIBITORY EFFECT OF DEFEROXAMINE ON *Paracoccidioides brasiliensis* SURVIVAL IN HUMAN MONOCYTES: REVERSAL BY HOLOTRANSFERRIN NOT BY APOTRANSFERRIN

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SUMMARY

The mechanisms used by *Paracoccidioides brasiliensis* to survive into phagocytic cells are not clear. Cellular iron metabolism is of critical importance to the growth of several intracellular pathogens whose capacity to multiply in mononuclear phagocytes is dependent on the availability of intracellular iron. Thus, the objective of this paper was to investigate the role of intracellular iron in regulating the capacity of *P. brasiliensis* yeast cells to survive within human monocytes. Treatment of monocytes with deferoxamine, an iron chelator, suppressed the survival of yeasts in a concentration-dependent manner. The effect of deferoxamine was reversed by iron-saturated transferrin (holotransferrin) but not by nonsaturated transferrin (apotransferrin). These results strongly suggest that *P. brasiliensis* survival in human monocytes is iron dependent.

KEYWORDS: *Paracoccidioides brasiliensis;* Monocytes; Iron; Deferoxamine.

INTRODUCTION

Paracoccidioidomycosis is a deep mycosis that is endemic in Latin America. This disease is caused by *Paracoccidioides brasiliensis*, a fungus that undergoes thermal dimorphism, developing as yeast at body temperature and as a mycelium at room temperature[5]. This fungus causes natural infections by inhalation of conidia or mycelial elements. These conidia convert to the parasitic yeast form in the lungs of mice[12]. Ingest conidia or yeast-forms of *P. brasiliensis* readily multiplies inside murine alveolar or peritoneal macrophages; however if they were activated by cytokines, such as IFN-γ, the multiplication was limited and conidia or yeast cells were killed[2,4]. Similarly, the interaction of human phagocytic cells and yeast-form of *P. brasiliensis* was studied *in vitro.* Yeast cells were readily ingested by monocytes or monocyte-derived macrophages. However, these cells allow *P. brasiliensis* multiplication, unless they were activated with IFN-γ or culture supernatants from concanavalin-A-stimulated mononuclear cells[13]. The mechanisms used by *P. brasiliensis* yeasts to survive and to multiply within human monocytes or macrophages are poorly understood. Iron has been considered an essential element for microbial growth and has also been associated with microorganisms' virulence by promoting intracellular growth[10]. Moreover, studies showed that both mycelial and yeast forms of *P. brasiliensis* have metabolic requirement for iron, which is shown in the ability of both forms to grow in the presence of low concentrations of iron[1]. In this study we have explored the role of iron in the intracellular growth or survival of *P. brasiliensis* in nonactivated human monocytes. We shall show that *P. brasiliensis* intracellular survival is iron dependent since this process is inhibited by iron chelator deferoxamine. Besides, the inhibition of deferoxamine is reverted by holotransferrin, an iron-saturated transferrin.

MATERIALS AND METHODS

1. Donors: We studied monocytes from healthy blood volunteer's donors, with informed consent from the University Hospital of the Botucatu Medical School, São Paulo State University (Brazil). This study was approved by the Ethics Committee of the Hospital.

2. Isolation of peripheral blood mononuclear cells: Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation at 300 × g for 30 min on Histopaque (d = 1.077) (Sigma Chemical Co., St. Louis, Mo.). Briefly, 10 mL of heparinized blood was mixed with an equal volume of complete tissue culture medium (CTCM) which consisted of RPMI 1640 tissue culture medium that does not contain iron (Sigma Chemical Co, St Louis, Mo), supplemented with 2 mM L-glutamine, 10% heat-inactivated human AB serum, 20 mM HEPES and 40 µg/mL gentamicin (Gibco Laboratories, Grand Island, N.Y.). Samples were layered over 5 mL of Histopaque in a 15 mL conical plastic centrifuge tube. After centrifugation at 300 × g for 30 min at room temperature, the interface layer of PBMC was harvested and washed twice with PBS-EDTA and once with CTCM. The counting of the monocytes were made with neutral red (0.02%) staining and the mononuclear cells were suspended to 2×10^6 monocytes/mL in CTCM.

3. Monocyte monolayers: The PBMC suspension, containing 2×10^6 monocytes/mL, was dispensed into 100 µL/wells in 96-well flat-bottomed plates (Nunc, Life Tech. Inc., Maryland, USA). After

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incubation for two h at 37 °C in 5% CO_2, nonadherent cells were removed by aspiration and each well was rinsed twice with CTCM. After adherence, the monocytes were cultured in CTCM at 37 °C in 5% CO_2 for 24 h, alone or containing the following treatments: a) deferoxamine (15, 25 and 35 µM), b) deferoxamine (35 µM) plus holotransferrin (3 and 6 µg/mL), c) deferoxamine (35 µM) plus apotransferrin (3 and 6 mg/mL). All these drugs were obtained from the Sigma Chemical Co. (St Louis, Mo).

4. Fungi: *P. brasiliensis* strain 18 (Pb 18) was maintained in the yeast-form cells at 35 °C on 2% Glucose, 1% Peptone, 0.5% Yeast extract and 2% agar medium (GPY medium) for six days. Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted as viable, while dark ones were considered not viable. Fungal suspensions containing more than 90% viable cells were used in the experiments.

5. Challenge of monocyte cultures with *P. brasiliensis* for evaluation of viable fungi recovery percentage: After removal of the supernatants from control (no treated) and treated monolayers, the viable fungus recovery of monocytes was tested by challenging with 2×10^4 viable yeasts/mL of Pb 18 (ratio 100 monocytes: 1 fungus) prepared with CTCM containing 10% fresh human AB serum, as source of complement for yeast opsonization. The control of phagocytosis was made by plating the supernatants of cocultures. After coculture for 18 h, cells were harvested by aspiration with sterile distilled water to lyse monocytes. Each culture well washing was contained in a final volume of 1 mL. The number of colony forming units (CFU) of *P. brasiliensis* in the cultures was determined by plating 100 µL of the 1 mL harvested volume on to brain-heart infusion agar medium (Difco Laboratories, Detroit, MI, USA) containing 4% normal horse serum and 5% *P. brasiliensis* strain 192 culture filtrate (v/v), the latter being the source of growth-promoting factor[16]. A well containing only 100 µL of *P. brasiliensis* yeast-form (2×10^4 viable units/mL) was also incubated using the same procedures for the monocyte cultures challenged with *P. brasiliensis*, to establish the calculation of viable fungi recovery percentage. Inoculated plates were incubated at 35 °C in sealed plastic bags to prevent drying. After 10 days the number of colony forming units (CFU) in each plate was counted. The percentage of viable fungus recovery was determined by the formula:

$$\text{Viable fungus recovery \%} = \frac{\text{CFU Mean of monocyte cultures (treated or not) challenged with } \textit{P. brasiliensis}}{\text{CFU Mean of } \textit{P. brasiliensis} \text{ culture}} \times 100$$

6. Statistical analysis: Data were statistically analyzed using the Sigma Stat 2.0 statistical software (Jandel Corporation). The results were compared by Friedman Test, with the level of significance set at $p < 0.05$.

RESULTS

Deferoxamine inhibits the intracellular survival of *P. brasiliensis* in human monocytes: In initial experiments we tested the effect of different concentrations of deferoxamine in the survival of *P. brasiliensis* within human monocytes. Monocyte monolayers were cultured for 24 h at 37 °C in the presence of three concentrations of deferoxamine (15, 25 and 35 µM) and after challenged with 2×10^3 yeasts. The recovery of viable fungi was detected by cocultures plating and counting of colony forming units (CFU) as described in Materials and Methods. Deferoxamine significantly inhibited the survival of the fungus within monocytes in the concentration of 35 µM (Fig. 1). The viability of control and deferoxamine-treated monocytes was > 95% as determined by trypan blue dye exclusion.

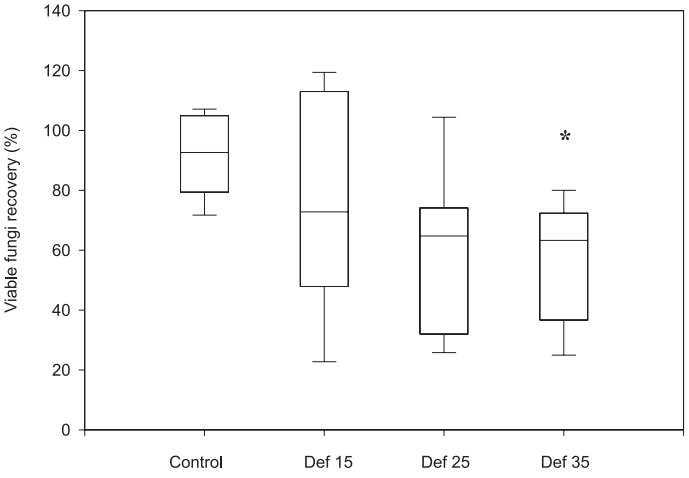

Fig. 1 - Deferoxamine inhibits the survival of *P. brasiliensis* yeasts in human monocytes in a concentration of 35 µM. Monocyte cultures were preincubated with deferoxamine at 15, 25 and 35 µM during 24 h and after challenged with 2 x 10^3 yeasts of *P. brasiliensis* during 18 h. The cocultures were plated and after 10 days the colony forming units (CFU) were counted. A culture containing only *P. brasiliensis* was also plated in the same conditions and the viable fungi recovery % was calculated. Box-and-whisker plot showing data distribution of 12 individuals. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles and vertical lines the 10th to 90th percentiles. * Statistical significance between groups is indicated - Control versus Def 35 ($p < 0.05$).

Holotransferrin reverses deferoxamine inhibition of intracellular survival of *P. brasiliensis* yeasts: To demonstrate that deferoxamine suppressed the intracellular survival of Pb18 yeasts by chelating iron from cells, human monocyte monolayers were cultured for 24 h at 37 °C in CTCM or in the presence of 35 µM of deferoxamine alone or with deferoxamine plus holotransferrin at 3 and 6 mg/mL. After, the cultures were challenged during 18 h with 2×10^3 yeasts of *P. brasiliensis.* The viable fungi recovery was detected by cocultures plating and colony forming units counting (CFU). Holotransferrin at 6 mg/mL, that replaced intracellular iron, completely reversed the inhibition of yeast survival by deferoxamine, and holotransferrin at 3 mg/mL partially reversed the effect of the chelator (Fig. 2).

Apotransferrin does not reverse deferoxamine inhibition of intracellular survival of *P. brasiliensis* yeasts: The monolayer treatment with deferoxamine plus apotransferrin (iron nonsaturated transferrin) at 3 and 6 mg/mL did not reverse the deferoxamine effect on the Pb18 survival, showing that the effect was not transferrin dependent, and the inhibition caused by deferoxamine on the survival of Pb18 in monocytes was really caused by chelate iron from the cell (Fig. 3).

DISCUSSION

Our study demonstrated that deferoxamine inhibits the intracellular survival of *P. brasiliensis* in human monocytes by interfering in the

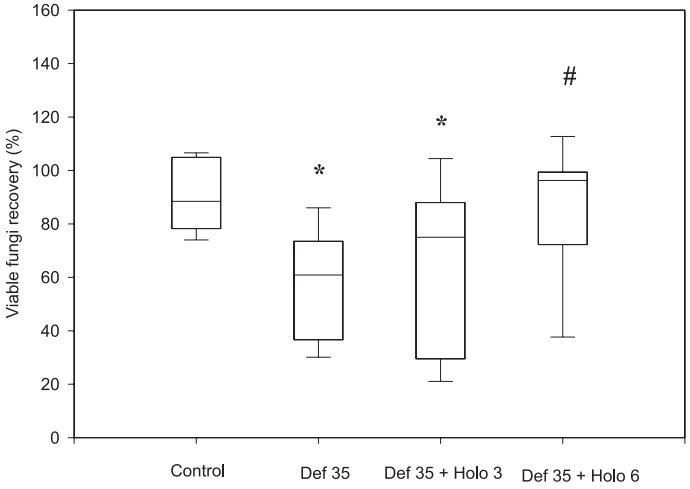

Fig. 2 - Holotransferrin completely reverses deferoxamine (35 µM) inhibition on the survival of Pb yeasts in human monocytes in a concentration of 6 mg/mL. Monocyte cultures were preincubated with deferoxamine plus holotransferrin (3 and 6 mg/mL) during 24 h and after challenged with 2 x 10^5 yeasts of *P. brasiliensis* during 18 h. The cocultures were plated and after 10 days the colony forming units (CFU) were counted. A culture containing only *P. brasiliensis* was also plated in the same conditions and the viable fungi recovery % was calculated. Box-and-whisker plot showing the median values of 12 individuals. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles and vertical lines the 10th to 90th percentiles. * Control versus Def 35 ($p < 0.05$); * Control versus Def 35+Holo3 ($p < 0.05$); # Def 35 versus Def 35+Holo6 ($p < 0.05$).

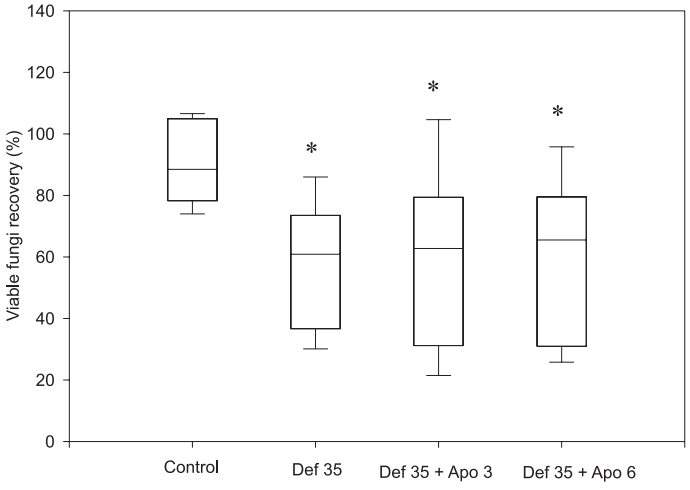

Fig. 3 - Apotransferrin does not reverse deferoxamine (35 µM) inhibition on the survival of Pb yeasts in human monocyte. Monocyte cultures were preincubated with deferoxamine plus apotransferrin (3 and 6 mg/mL) during 24 h and after challenged with 2 x 10^5 yeasts of *P. brasiliensis* during 18 h. The cocultures were plated and after 10 days the colony forming units (CFU) were counted. A culture containing only *P. brasiliensis* was also plated in the same conditions and the viable fungi recovery % was calculated. Box-and-whisker plot showing the median values of 12 individuals. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles and vertical lines the 10th to 90th percentiles. * Control versus Def 35 ($p < 0.05$); * Control versus Def 35+Apo3 ($p < 0.05$); * Control versus Def 35+Apo6 ($p < 0.05$).

iron acquisition, reinforcing the hypothesis that the availability of intracellular iron is clearly required for the survival of *P. brasiliensis* yeast cells in human phagocytic cells. The role of iron in the process above is based upon the experiments showing that during infections a decrease in serum free iron associated with an increase of serum transferrin and lactoferrin occur[14,18,19,20]. Thus, iron is accumulated in spleen and liver macrophages by inhibition of its release from these cells, preventing its normal recycling to transferrin[21]. Concomitantly, macrophages initiate the ferritin synthesis that enables these cells to acquire iron from ingested erythrocytes. Paradoxically, this response to infection may enhance the pathogenicity of intracellular microorganisms such as *P. brasiliensis*, by providing them sufficient amount of iron, which is important for their survival or multiplication inside these cells.

Although we did not analyze the influence of deferoxamine in the phagocytic indices, due to the difficulties in standardizing this type of assay with *P. brasiliensis* yeast cells, we plated all coculture supernatants in order to verify if there were free yeasts in the culture. We did not recover significant amounts of CFU in these plates showing that our opsonization system using fresh AB human serum was effective. These results allow us to consider that the deferoxamine effect on the yeast survival decrease was not due to impairment of phagocytosis. Besides, other authors verified that deferoxamine did not influence phagocytosis of opsonized sheep erythrocytes and the microscopic inspection failed to show any significant morphological differences between deferoxamine-treated and no treated cells[11].

In our assays, we observed a reduction of *P. brasiliensis* survival after deferoxamine treatment. This finding may be explained because this drug, under physiologic conditions, does not chelate a significant amount of iron bound to transferrin[15], and does not remove it from ferritin[8]. Thus, deferoxamine chelates only the free iron pool, suggesting that *P. brasiliensis* takes it from the intracellular pool, what can be used by the cell or by microorganisms. In the present study, *P. brasiliensis* survival inhibition was reversed by holotransferrin, which, in turn, could supply sufficient iron, and then, revert deferoxamine effects. This result was expected seeing that holotransferrin contains two molecules of ferric iron, and when it binds to the transferrin receptor, this complex is endocytosed. Within the cell, the endocytic vacuole is acidified, and transferrin releases ferric ions, which is incorporated to the available iron pool, used by the cell or microorganisms[7,17]. Our result with apotransferrin plus deferoxamine treatment showed that the effects were not mediated by transferrin.

The modulation of intracellular iron pool by deferoxamine has been studied in the interaction of other microorganisms with phagocytic cells. The treatment of phagocytic cells with deferoxamine inhibited *H. capsulatum*[9], *T. cruzi*[11] and *L. pneumophila*[3] replication. This same effect was seen preincubating these cells with IFN-γ, which reduced transferrin receptors expression, and also limited the available intracellular iron[3,9]. The effect of deferoxamine and IFN-γ was reversed by simultaneous incubation with holotransferrin, which replaced intracellular iron. On the contrary, *L. pneumophila* growth increased in tioglicolate-stimulated murine peritoneal macrophages, and this process was associated with higher transferrin receptor expression and increased free intracellular iron. Either apotransferrin or deferoxamine inhibited this intracellular growth by down regulating the intracellular iron pool, being reversed by holotransferrin or FENTA (ferric nitrilotriacetate)[6].

In conclusion, our study strongly supports the hypothesis that *P. brasiliensis* survival in human monocytes is iron dependant. As mentioned, several studies demonstrated that the phagocytic activation process, mainly the one that is IFN-γ dependant, inhibits transferrin receptor expression with consequent reduction of the iron pool labile. Thus, in paracoccidioidomycosis patients, alterations in cytokines levels, that could either interfere in the transferrin receptor or other mechanisms involved in iron availability, could favor *P. brasiliensis* survival or growth in phagocytic cells patients. Future investigations may better elucidate these mechanisms.

RESUMO

Efeito inibidor da deferoxamina sobre a sobrevivência do *Paracoccidioides brasiliensis* em monócitos humanos: reversão por holotransferrina e não por apotransferrina

Os mecanismos utilizados pelo *Paracoccidioides brasiliensis* para sobreviver em células fagocitárias ainda não estão elucidados. O metabolismo celular férrico é muito importante para o crescimento de inúmeros patógenos intracelulares cuja capacidade de se multiplicarem em fagócitos mononucleares é dependente da disponibilidade intracelular do íon ferro. Assim, o objetivo deste trabalho foi investigar o papel do ferro intracelular sobre a capacidade do *P. brasiliensis* sobreviver em monócitos humanos. O tratamento de monócitos com deferoxamina, uma droga quelante, diminuiu a sobrevivência de leveduras do fungo de forma dose-dependente. O efeito inibidor da deferoxamina sobre a sobrevivência do *P. brasiliensis* foi revertido por transferrina saturada com ferro (holotransferrina) mas não por transferrina insaturada (apotransferrina). Estes resultados sugerem que a sobrevivência do *P. brasiliensis* em monócitos humanos é dependente do íon ferro.

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