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Inhibitory Effect of Deferoxamine or Macrophage Activation on Transformation of *Paracoccidioides brasiliensis* Conidia Ingested by Macrophages: Reversal by Holotransferrin

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Conidia of *P. brasiliensis* ingested by murine macrophages at 37°C showed enhanced transformation to yeast cells and further intracellular growth compared with conidia in culture medium alone. Treatment of macrophages with the iron chelator deferoxamine inhibited the intracellular conidium-to-yeast transformation. Cytokine-activated macrophages could also exert this inhibitory effect. Holotransferrin reversed the inhibitory effect of either deferoxamine or activated macrophages on intracellular conidium-to-yeast transformation. These results indicate that iron restriction is one of the mechanisms by which activated macrophages control the intracellular transformation of ingested conidia and growth of yeast cells.

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, the most common systemic mycosis in Latin America (2, 18). Natural infection begins with inhalation of conidia or mycelia fragments produced by the saprophytic phase of this thermally dimorphic fungal pathogen (2, 18). Histological studies have revealed that inhaled conidia quickly convert to the yeast form in the lungs of experimentally infected mice (16).

Murine pulmonary and peritoneal macrophages can be activated in vitro by cytokines (CK) or gamma interferon for fungicidal activity against yeast form *P. brasiliensis* (3, 5). Conidia ingested by murine peritoneal macrophages had enhanced frequency of transformation to yeast cells compared with conidia in culture medium alone (10). The yeast cells subsequently grew intracellularly by budding. Macrophages activated by CK significantly inhibited transformation of ingested conidia (10).

In this study we explored the role of iron on intracellular transformation of conidia to yeast cells in resident or activated macrophages.

Conidia. Conidia of isolate Gra (ATCC 60855) were obtained as previously described (19). Viability was assessed by the ethidium bromide-fluorescein diacetate method (9, 17), and this correlated with conidial ability to germinate in the agar-microscope slide assay (10). Viability of conidia was always greater than 80%.

Peritoneal macrophages. Adherent macrophages from male BALB/c mice were obtained as previously described (5, 10). Briefly, 0.2 ml of 2×10^6 peritoneal cells per ml of complete tissue medium (CTCM), consisting of RPMI 1640 medium plus 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U of penicillin, and 100 μg of streptomycin per ml (GIBCO Laboratories, Grand Island, N.Y.), was dispensed into each 1-cm^2 chamber of eight-chambered Lab-Tek slides (Miles Scientific, Naperville, Ill.). After 2 h at 37°C in 5% CO_2 and 95% air, nonadherent cells were removed. Approximately 2×10^5 adherent macrophages per chamber formed a monolayer (3).

Immunization and CK. BALB/c mice were immunized intravenously with 10^6 heat-killed conidia as previously described (10). Spleen cell suspensions from immunized or nonimmunized mice were prepared and purified by density gradient centrifugation (10). Spleen cells from immunized and nonimmunized mice were suspended to 5×10^6 per ml of CTCM and stimulated with *P. brasiliensis* antigen (10) in CTCM; controls were treated identically but without antigen. After incubation for 72 h at 37°C in 5% CO_2 and 95% air, supernatants were collected and filtered (0.45-μm-pore filter), and portions were stored at −20°C.

Treatment of macrophages. Macrophages have receptors for transferrin, the iron-transporting protein in serum. Macrophage monolayers were treated by removing the supernatant and replacing it with a 0.25-ml solution containing the following entities alone or in combination: CTCM, CK from immune spleen cells (C-ICK) or from immune cells stimulated with antigen (Ag-ICK), deferoxamine mesylate (DEX) (CIBA Pharmaceutical Company, Summit, N.J.), human holotransferrin (iron-saturated transferrin) (HOLO), and apotransferrin (iron-free protein) (APO) (Miles Laboratories, Naperville, Ill.). Overnight treatment was done at 37°C in 5% CO_2 and 95% air. DEX at 25 μM did not affect viability of the macrophages as assessed by trypan blue dye exclusion test.

Infection of macrophages. Conidia (10^6) were suspended in 10 ml of RPMI 1640 plus 30% (vol/vol) fresh mouse serum from nonimmunized mice. The suspension was incubated at 37°C for 20 min to allow for opsonization (8). Opsonized conidia were suspended at 10^5/ml in CTCM, CK supernatants, and/or deferoxamine. Macrophage monolayers were infected with 0.2 ml of conidial suspensions, which gave a conidium-to-macrophage ratio of 1:10. Within 4 h more than 80% of the conidia were phagocytosed by the variously treated macrophage monolayers.

Time course measurements. Lab-Tek slide cultures were

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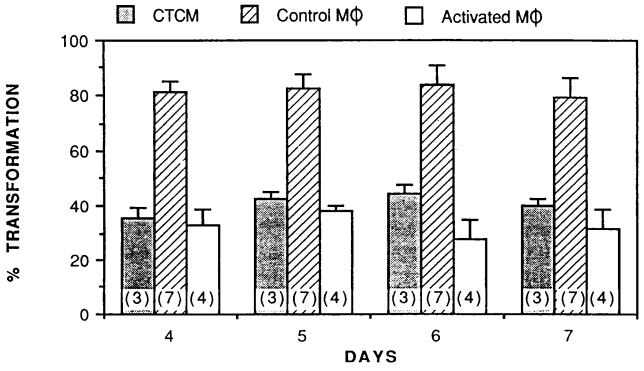

FIG. 1. Enhanced conidium-to-yeast transformation in CTCM-cultured macrophages (Mϕ). Bars represent the mean percent transformation of conidia to yeast cells in CTCM alone (speckled bars), CTCM-cultured macrophages (slashed bars), and cytokine-treated macrophages (open bars) after 4, 5, 6, and 7 days. The number of experiments is shown in parentheses, and the standard deviations from the mean are indicated by vertical lines.

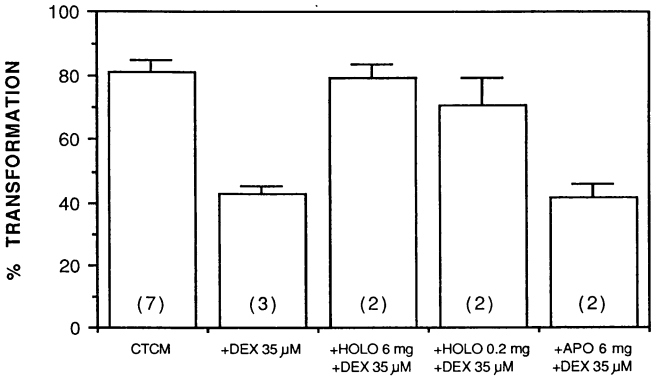

FIG. 2. Effect of DEX, HOLO, and APO on transformation of conidia to yeast cells in CTCM-cultured macrophages. Bars indicate mean percent transformation in 7 days of conidia to yeast cells in CTCM-cultured macrophages treated with DEX, HOLO plus DEX, or APO plus DEX. The number in parentheses is the number of experiments, and vertical lines show the standard deviations from the mean.

incubated in a humidified incubator at 37°C in 5% CO_2 and 95% air. Since most transformation begins only by day 4 (10), observations began at this time. Sets of cultures were processed on days 4, 5, 6, and 7 by aspirating the medium, washing with phosphate-buffered saline, drying in air, and staining with the Diff-Quik method (American Scientific Products, McGaw Park, Ill.). At these times extracellular fungi were rarely seen. Macrophages incubated for 7 days without a change of medium retained their morphology, and their viability was stable as assessed by trypan blue exclusion. Over 200 macrophages with intracellular *P. brasiliensis* were examined per monolayer, and conidia or yeast cells in macrophages were recorded. Differentiation between these two forms of *P. brasiliensis* is easy because of size and morphological differences, as shown in previous reports (10, 20).

Statistical analysis. Comparisons between groups were analyzed by the Student *t* test, with significance assumed to be $P < 0.05$.

There was enhanced transformation of conidia to yeast cells in macrophages treated with CTCM or C-ICK (data not shown) compared with that in Ag-ICK treated macrophages or in CTCM alone (Fig. 1). The effect seen by day 4 was maintained through day 7 and was significant at these times ($P < 0.001$).

When CTCM-cultured macrophages were treated with various concentrations of DEX (0.5, 5, 15, 25, or 35 μM) for 24 h and then infected with opsonized conidia plus DEX, the intracellular transformation of conidia to yeast cells was significantly ($P < 0.01$) inhibited by 15 to 35 μM DEX at days 4, 5, 6, and 7 (data not shown). At day 4, DEX at 35 μM was more inhibitory than at 15 μM (38.0% ± 7.0% versus 26.4% ± 5.7%), but this was not significant ($P > 0.05$).

The inhibitory effect of DEX on intracellular transformation of conidia to yeast cells in CTCM-cultured macrophages was reversed ($P < 0.01$) by HOLO (0.2 and 6 mg/ml) but not by APO (6 mg/ml) (Fig. 2). This indicated that iron-saturated transferrin could supply iron sufficient to overcome the effects of DEX and still support iron requirements for conidium-to-yeast transformation.

The inhibitory effect of activated (Ag-ICK-treated) macrophages on intracellular conidium-to-yeast transformation was also reversed ($P < 0.001$) by HOLO (0.2 or 6 mg/ml) but not by APO (6 mg/ml) (Fig. 3). This suggests that activated macrophages did not kill conidia. These results suggest that for the conidia, activated macrophages were an environment deficient in utilizable iron and that HOLO could supply the iron required for efficient intracellular conidium-to-yeast transformation.

Although there is evidence that *P. brasiliensis* produces siderophores (11), their insufficient concentration or lack of ability to remove iron from transferrin at neutral pH could account for poor conidium-to-yeast transformation in CTCM alone (10) or poor growth of yeast cells in CTCM (4). On the other hand, macrophages can make utilizable iron available to ingested conidia and yeast cells by the transferrin-transferrin receptor transport system. The transferrin-transferrin receptor complex is endocytized, and upon acidification, ferric iron dissociates from transferrin and enters the labile iron pool (7). DEX can chelate iron in the labile iron pool (15) and consequently inhibit conidium-to-yeast transformation by iron

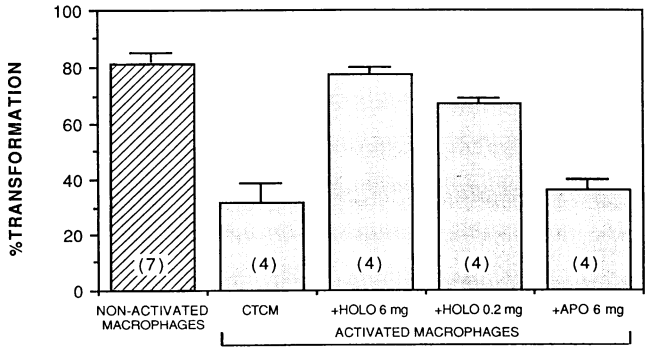

FIG. 3. Inhibition of conidium-to-yeast transformation by activated (Ag-ICK treated) macrophages reversed by HOLO. Percent transformation of conidia to yeast cells in CTCM-cultured macrophages (slashed bar) by day 7 is shown. The effect of CTCM, HOLO, or APO on activated macrophage inhibition of conidium-to-yeast transformation is indicated by speckled bars. The number of experiments is shown in parentheses, and the standard deviations from the mean are shown as vertical lines.

deprivation. Our results show that iron-saturated transferrin can overcome the effects of DEX.

Others have shown that transferrin receptors are down regulated on activated macrophages (12, 13) and the antimicrobial effects of activated macrophages against certain intracellular pathogens are mediated by iron deprivation (1, 6, 14). As we have shown here, and others have shown (1), iron-saturated transferrin can compensate for the reduced number of transferrin receptors on activated macrophages by getting enough iron transported into the activated macrophage to support the intracellular pathogen and reverse the antimicrobial effect of transferrin receptor down regulation.

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