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Fate of Conidia of *Paracoccidioides brasiliensis* after Ingestion by Resident Macrophages or Cytokine-Treated Macrophages

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Conidia ingested by resident macrophages had an enhanced percentage of transformation to yeast cells compared with those in culture medium without macrophages. The yeast cells subsequently grew intracellularly by budding. Macrophages treated with cytokines from antigen-stimulated spleen cells from immunized mice significantly inhibited transformation of ingested conidia.

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, the most common systemic mycosis in Latin America (10). Natural infections start by inhalation of conidia or other elements of the saprophytic phase of this thermally dimorphic fungal pathogen (10). Histological studies show that conidia quickly convert to the yeast form in the lungs of infected mice (9). Although it has been demonstrated that murine pulmonary or peritoneal macrophages can be activated by gamma interferon for fungicidal activity against the yeast form in vitro (3, 4), little is known about interaction of the infectious propagules (conidia) and resident or activated macrophages. Moreover, it is not known whether conidia can transform into yeast cells and grow intracellularly after ingestion by macrophages.

The purpose of the present study was to determine (i) whether conidia transform into the yeast form more efficiently extracellularly or intracellularly after ingestion by macrophages and (ii) the fate of conidia ingested by cytokine-treated macrophages.

Conidia. A patient isolate of *P. brasiliensis* (Gra, ATCC 60855) was previously shown to conidiate on glucose-salts agar (13). Conidia produced by this method were used in all experiments. Washed conidia were counted with a hemacytometer, and viability was assessed by using ethidium bromide-fluorescein diacetate (7, 11). Results obtained with this method correlate with conidial ability to germinate in the agar-microscope slide assay (8). Viability of conidia was always >80%. The composition of conidial inocula was as previously reported (9), with no yeasts and <13% mycelial fragments; the morphology and characterization of the conidia have been previously described (9, 14).

Peritoneal macrophages. Peritoneal cells were obtained by lavage of the abdominal cavity (4) of 12-week-old male BALB/c mice. The mice were from the breeding colony at Corporacion para Investigaciones Biologicas. Peritoneal cells were washed once and suspended at 2×10^6 cells per ml of complete tissue culture medium (CTCM). CTCM consisted of RPMI 1640 containing 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (GIBCO Laboratories, Grand Island, N.Y.). A 0.2-ml volume of peritoneal cells was dispensed into each chamber of eight-chambered Lab-Tek slides (Miles Scientific, Naperville, Ill.). Cultures were incubated at 37°C in 5% CO_2–95% air for 2 h, and then nonadherent cells were removed by aspiration and adherent monolayers were rinsed with RPMI 1640. Approximately 50 to 60% of seeded cells adhered and formed a monolayer (2×10^5 adherent cells per chamber) (4).

Immunization. BALB/c mice were immunized intravenously with 10^6 heat-killed conidia suspended in phosphate-buffered saline (PBS) (pH 7.2). Sensitization to antigen was confirmed 2 weeks later by testing for delayed-type hypersensitivity as measured by footpad swelling. A paracoccidioidal skin test antigen (12) (50 μg in 0.01 ml) was injected subcutaneously into one hind footpad, and 0.01 ml of PBS was injected into the other hind footpad (control). Antigen-induced footpad swelling was significantly different from that of PBS controls (a total of 22 animals; $P < 0.01$). Nonimmunized mice did not respond to the antigen by footpad swelling.

Cytokines. Spleen cell suspensions from immunized or nonimmunized mice were prepared, and cytokines were produced (2). Spleen cells were purified by density gradient centrifugation over Ficoll-Hypaque (density = 1.077) (Sigma Chemical Co., St. Louis, Mo.) at $400 \times g$ for 15 min. Mononuclear cells were harvested from the interface and washed twice with CTCM. Cells were suspended to 5×10^6/ml of CTCM and exposed to paracoccidioidal antigen (12) (50 μg/ml) or CTCM without antigen for 2 h at 37°C. Cultures were centrifuged ($400 \times g$, 10 min), supernatants were aspirated to remove excess antigen, and fresh medium without antigen was added. After incubation for 72 h at 37°C in 5% CO_2–95% air, cultures were centrifuged ($400 \times g$, 10 min), supernatants were collected and filtered (0.45 μm-pore-size filter), and portions were stored at −20°C. Control supernatants were produced by incubating spleen cells from nonimmunized mice with or without antigen.

Treatment of macrophages. Macrophage monolayers were treated with 0.25 ml of CTCM, cytokines in supernatants (CK) from antigen-stimulated immune spleen cells (Ag-ICK), CK from nonstimulated immune spleen cells (Nil-ICK), CK from antigen-stimulated nonimmune spleen cells (Ag-NCK), or CK from nonstimulated normal spleen cells (Nil-NCK). Macrophages were treated overnight at 37°C in 5% CO_2–95% air.

Infection of macrophages. Conidia were suspended in RPMI 1640 containing 30% (vol/vol) fresh mouse serum from

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nonimmunized mice at 10^6 conidia per ml. Conidial suspensions were incubated at 37°C for 20 min to allow opsonization to take place (6). Opsonized conidia were suspended in CTCM or supernatant at 10^5/ml. Macrophage monolayers were infected with 0.2 ml of the conidial suspension, which gave a conidium-to-macrophage ratio of 1:10. Within 4 h ≥80% of the conidia were phagocytosed by each of the variously treated monolayers.

Time course measurements. Lab-Tek slide cultures were incubated in a humidified incubator at 37°C in 5% CO_2–95% air. Sets of monolayer cultures were processed on days 4, 5, 6, and 7 (by these times extracellular fungi were rarely seen) by aspiration of medium, rinsing with PBS, drying in air, and staining with Diff-Quik (American Scientific Products, McGaw Park, Ill.). The monolayer media were not changed during the 7 days of incubation. The monolayers at day 7 retained normal morphology, and their viability was stable as assessed by trypan blue exclusion. Over 200 macrophages with intracellular *P. brasiliensis* were examined per monolayer, and the morphology, e.g., conidium, yeast cell, or multiple budding yeast, was recorded. These forms of *P. brasiliensis* are easily distinguished, as shown in a previous report (14). In the text and figures we use yeasts or yeast cells as shorthand for nonbudding single yeast cells. The low fungus-to-macrophage ratio made it likely that the fungi observed were intracellular rather than attached, and previous correlations between light microscopic and electron microscopic observations for which similar culture techniques were used confirm this (4, 5).

Statistics. Comparison between groups were analyzed by the Student *t* test, with the significance level assumed to be $P < 0.05$.

Transformation of conidia in medium. When conidia were incubated at 37°C in control supernatants (Nil-ICK) alone, transformation and development into multiply budding yeast cells were limited. After 4 to 7 days of incubation, cultures primarily contained conidia (60-65%), and the remaining forms consisted of about equal numbers of single and budding yeast cells (Fig. 1). Similar results were obtained when conidia were incubated with CTCM or supernatant (Ag-ICK, Ag-NCK, or Nil-NCK).

Intracellular transformation of conidia. Conidia ingested by macrophages treated with control supernatants (Nil-ICK) readily transformed intracellularly. After 4 days of incubation, intracellular *P. brasiliensis* consisted of 16% ± 2% conidia, 32% ± 11% single yeast cells, and 52% ± 13% budding yeast cells (Fig. 2). With time (7 days), 78% ± 2% of the intracellular forms consisted of multiply budding yeast cells (Fig. 2). When macrophages were incubated with other control supernatants (Ag-NCK or Nil-NCK) or with CTCM similar results were obtained. These findings demonstrate not only that conidia can transform and multiply inside macrophages but that this intracellular milieu greatly enhances transformation of conidia compared with that in medium alone. Comparison of the proportions of conidia transformed into multiply budding yeast cells when cultured in the absence of macrophages and when incubated with Nil-ICK-treated macrophages (Fig. 1 and 2) indicated that the former transformed poorly (approximately 20%) while the latter transformed at a percentage two- to fourfold greater, irrespective of the time of incubation ($P < 0.005$ for day 4; $P < 0.0005$ for day 5, 6, or 7).

Fate of conidia in Ag-ICK-treated macrophages. Compared with control macrophages, Ag-ICK-treated macrophages significantly inhibited transformation of conidia and their further development. After 4 days of incubation 56% ± 9% of intracellular *P. brasiliensis* were conidia, and this did not change significantly with more days of incubation; e.g., 64% ± 3% were conidia at day 7 (Fig. 3). Although the percentage of single yeast cells (34% ± 4%) at day 4 (Fig. 3) was similar to that in control macrophages (32% ± 11%), the percentage of budding yeast cells (18% ± 3%) was significantly less ($P < 0.001$) than that in control macrophages (52% ± 13%). Thus, the percent transformed conidia (sum of single yeast cells and budding yeasts) was less in Ag-ICK-treated macrophages. Three different batches of supernatants prepared under identical conditions gave similar results. These results indicate that Ag-ICK-treated macrophages relative to control macrophages (macrophages treated with Nil-ICK, Ag-NCK, Nil-NCK, or CTCM) not only inhibited the transformation of conidia but also inhibited the intracellular growth (budding) of yeast cells from transformed conidia.

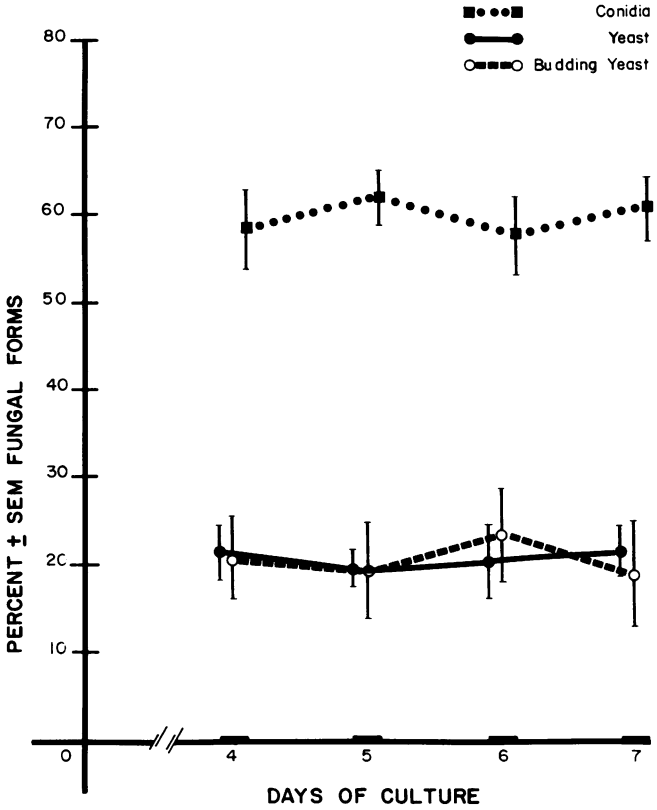

FIG. 1. Transformation of conidia in control supernatant alone. Shown are the mean percentages ± standard errors of the means (n = 3 experiments) of fungal forms after conidia were cultured in control supernatant alone for 4, 5, 6, or 7 days.

Effect of Ag-ICK dose on macrophages. When macrophages were treated with one of a series of twofold dilutions of Ag-ICK, induction of inhibitory activity in macrophages was dose dependent. Ag-ICK undiluted or diluted 1:2 were equally efficient in causing macrophages to inhibit germination of ingested conidia (Fig. 4a and b); e.g., 65 to 70% of the ingested conidia failed to transform. The inhibitory activity of macrophages dropped dramatically when they were treated with Ag-ICK diluted 1:4 or 1:8. Most (75%) of the conidia transformed, and 40 to 50% were budding yeast cells (Fig. 4c and d).

We showed that conidia of *P. brasiliensis* transformed poorly under conditions that were optimal for culturing

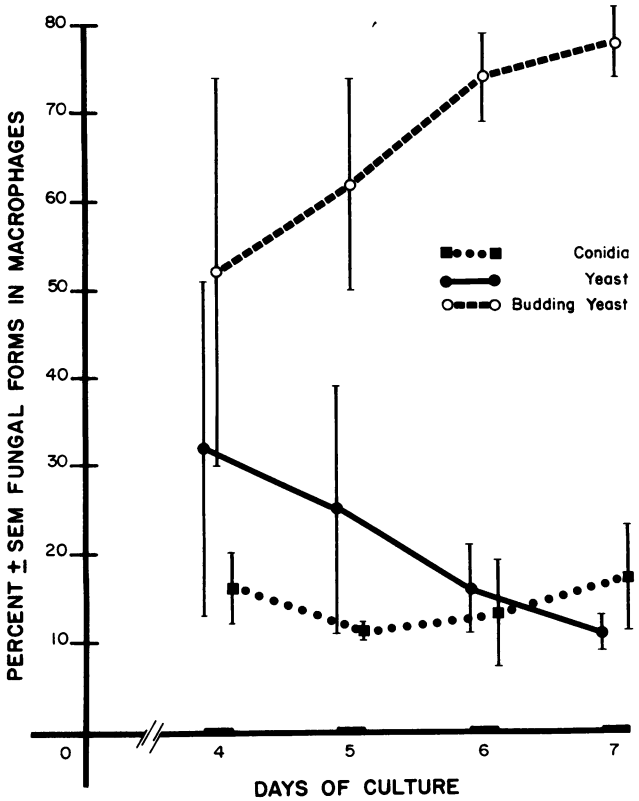

FIG. 2. Transformation of conidia in macrophages treated with control supernatants. Shown are mean percentages ± standard errors of the means of macrophages containing conidia, yeast cells, or budding yeast cells after 4, 5, 6, or 7 days. Each point represents the mean from three separate experiments.

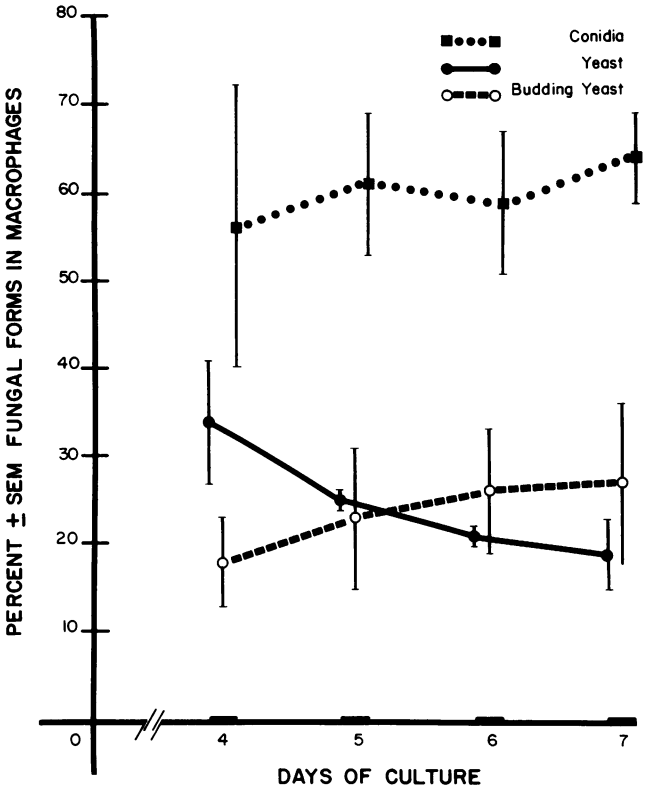

FIG. 3. Inhibition of intracellular germination of conidia by macrophages treated with Ag-ICK. Shown are mean percentages ± standard errors of the means of macrophages containing conidia, yeast cells, or budding yeast cells after 4, 5, 6, or 7 days of culture. Combined results from three separate experiments are shown.

macrophages. For example, in CTCM, there was a 3- to 4-day lag period before an appreciable number of conidia transformed into yeast cells. Whether this is also true for extracellular conditions encountered during natural infection, e.g., bronchi or alveolar spaces in the lung, is not known. Conidia can transform and produce budding yeast cells in the lungs of infected mice in a relatively short time (9), e.g., 36 h. However, these histological studies (9) were not able to determine whether transformation took place extracellularly, intracellularly, or both. The transformation time was similar to that for transformation of conidia into yeast cells upon incubation in Mueller-Hinton agar at 37°C (14). The factors present in vivo (lungs) and in Mueller-Hinton medium that accelerate transformation of conidia into yeast cells, but which are apparently lacking in CTCM, are unclear at this time.

Transformation of conidia was significantly enhanced when they were ingested by control or normal macrophages. Moreover, yeast cells from transformed conidia grew intracellularly by budding, compared with slowed fungal growth in control medium alone. These findings are in agreement with previous work which showed, by measurement of CFU, that the intracellular milieu of normal macrophages relative to serum-supplemented tissue culture medium favors the growth of ingested yeast cells (4). Thus, the morphological observation of a lack of an increased percentage of multiply budding cells compared with yeasts is consistent with fungistasis, and conversely, an increase in the percentage of multiply budding cells is consistent with fungal growth. Our data suggest that phagocytosis of conidia by resident macrophages may not be protective but on the contrary may favor the establishment of infection.

In the present study, it was shown that macrophages treated with cytokines generated by antigen stimulation of sensitized spleen cells (Ag-ICK) induced macrophage monolayers to significantly inhibit the transformation of ingested conidia and their further development by budding. This is consistent with a previous study (4), in which CFU were measured, which showed not only restriction of yeast multiplication in activated macrophages but also reduction of CFU (killing). Our experiments did not determine whether Ag-ICK-treated macrophages (or a subpopulation thereof) killed a percentage of the conidia or whether the mechanism was purely fungistatic in a subpopulation of the monolayer. Preliminary results with pulmonary macrophages and conidia from another isolate (Cob) suggest that the first possibility may be the case (8). Moreover, the data suggest that at least a subpopulation of macrophages did not respond, or fully respond, to Ag-ICK, because some macrophages in the monolayer supported the transformation of ingested conidia.

It is possible that Ag-ICK contained gamma interferon, an activator of macrophages for fungicidal activity (3, 4), and that this was the active moiety in Ag-ICK. This interpretation is consistent with work done with another dimorphic fungal pathogen, *Coccidioides immitis* (1, 2); gamma inter-

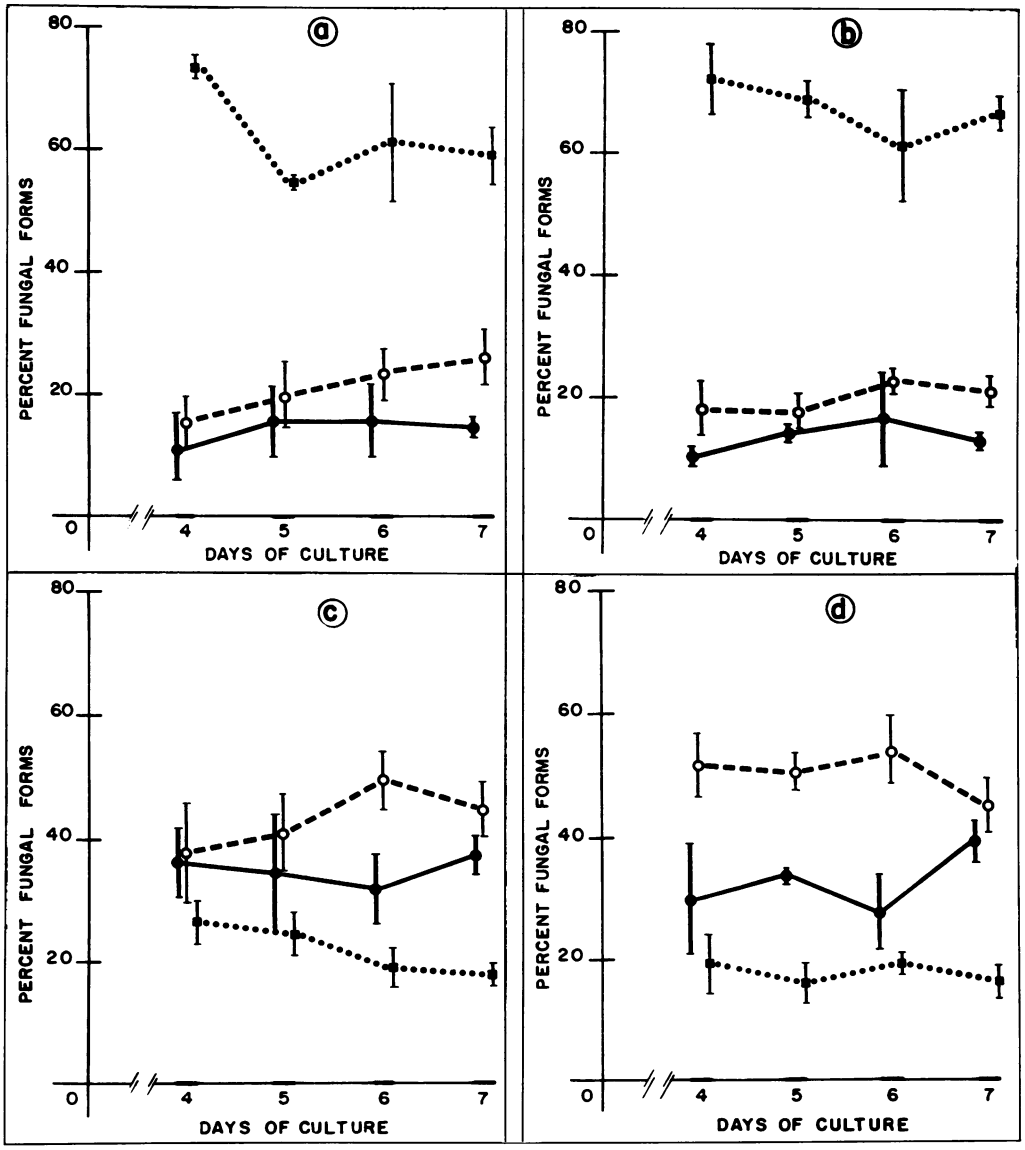

FIG. 4. Effect of Ag-ICK dose on macrophage inhibitory activity. Shown are mean percentages ± standard errors of the means (n = 3 experiments) of macrophages containing conidia (squares), yeast cells (solid circles), or budding yeast cells (open circles) after treatment with Ag-ICK at a dilution of 1:1 (a), 1:2 (b), 1:4 (c), or 1:8 (d). Results from 4-, 5-, 6-, and 7-day cultures are shown.

feron or supernatants from antigen-stimulated spleen cells from immunized mice activated macrophages to inhibit the transformation of ingested arthroconidia. In another, similar study (8), supernatants, known to contain gamma interferon and macrophage inhibitory factor, from concanavalin A-stimulated spleen cells of nonimmune mice had activity similar to that of supernatants from antigen-stimulated immune spleen cells. The present study provides a rationale for further work with purified cytokines, such as cytokines prepared by recombinant techniques, in the study of *Paracoccidioides*-effector cell interactions to define the active moieties and their possible use in immunotherapy.

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