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Intracellular Multiplication of *Paracoccidioides brasiliensis* in Macrophages: Killing and Restriction of Multiplication by Activated Macrophages

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The effect of coculturing yeast-form *Paracoccidioides brasiliensis* with murine cells was studied. Coculture of resident peritoneal or pulmonary macrophages with *P. brasiliensis* for 72 h dramatically enhanced fungal multiplication 19.3 ± 2.4- and 4.7 ± 0.8-fold, respectively, compared with cocultures with lymph node cells or complete tissue culture medium alone. Support of *P. brasiliensis* multiplication by resident peritoneal macrophages was macrophage dose dependent. Lysates of macrophages, supernatants from macrophage cultures, or McVeigh-Morton broth, like complete tissue culture medium, did not support multiplication of *P. brasiliensis* in 72-h cultures. Time course microscopic studies of cocultures in slide wells showed that macrophages ingested *P. brasiliensis* cells and that the ingested cells multiplied intracellularly. In sharp contrast to resident macrophages, lymphokine-activated peritoneal and pulmonary macrophages not only prevented multiplication but reduced inoculum CFU by 96 and 100%, respectively, in 72 h. Microscopic studies confirmed killing and digestion of *P. brasiliensis* ingested by activated macrophages in 48 h. These findings indicate that resident macrophages are permissive for intracellular multiplication of *P. brasiliensis* and that this could be a factor in pathogenicity. By contrast, activated macrophages are fungicidal for *P. brasiliensis*.

Paracoccidioides brasiliensis is the causative agent of human paracoccidioidomycosis, the most common systemic mycosis in Central and South America (12). Natural infections by this thermally dimorphic fungal pathogen start by inhalation of conidia or other elements of the saprophytic phase (12). Histological studies show that conidia convert to the yeast form in the lungs of mice within hours after infection (9). Although polymorphonuclear neutrophils and macrophages become involved in lesions within days after infection (9), polymorphonuclear neutrophils (14) and resident macrophages (2) do not appear to be fungicidal for *P. brasiliensis* in vitro. However, macrophages activated with gamma interferon significantly kill yeast-form *P. brasiliensis* (2, 3), as demonstrated in short-term (4-h) assays.

We have extended previous short-term assay studies to long-term studies of interactions between macrophages and *P. brasiliensis*. We report here evidence that resident macrophages are permissive for the intracellular multiplication of ingested *P. brasiliensis*, a phenomenon not appreciated up to this time. Resident macrophages, especially peritoneal macrophages, not only failed to restrict multiplication of *P. brasiliensis* in 72- and 144-h cocultures but significantly enhanced multiplication severalfold compared with cocultures with lymph node cells or complete tissue culture medium alone. Microscopic studies showed that *P. brasiliensis* multiplied intracellularly in macrophages. In contrast, lymphokine-activated macrophages were fungicidal for ingested *P. brasiliensis*.

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MATERIALS AND METHODS

Animals. Male BALB/cByJIMR mice, 8 to 12 weeks of age, were obtained from the Institute for Medical Research (San Jose, Calif.) for use in these experiments.

Reagents and media. Tissue culture medium RPMI 1640 with L-glutamine, Eagle minimum essential medium, heat-inactivated fetal bovine serum, and a penicillin (10,000 U/ml)–streptomycin (10,000 μg/ml) mixture were obtained from GIBCO Laboratories (Grand Island, N.Y.). Complete tissue culture medium (CTCM) consisted of 89 ml of RPMI 1640, 10 ml of fetal bovine serum, and 1 ml of penicillin-streptomycin. Concanavalin A (ConA) and α-methyl-D-mannoside (grade III) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Murine gamma interferon produced by recombinant DNA technology was characterized and supplied by Genentech Corporation (South San Francisco, Calif.) (16).

Fungi. *P. brasiliensis* isolates Gar APw, LA (ATCC 32074), and Gra (ATCC 60855) were from Colombian patients with paracoccidioidomycosis. Yeast-form cultures were maintained on brain heart infusion (BHI) agar slants at 35°C and transferred to BHI agar slants monthly. For inoculum preparation, *P. brasiliensis* was grown in modified McVeigh-Morton (MMvM) broth (13) (3 ml per 10-ml plastic tube [Falcon 2051; Becton-Dickinson and Co., Lincoln Park, N.J.]) at 35°C on a gyratory shaker (220 rpm) and subcultured weekly in MMvM broth. Viability of yeast cells was determined by using fluorescein diacetate-ethidium bromide vital staining (11). To determine CFU, a new culture medium with enhanced plating efficiency was used (7, 7a). It consisted of BHI agar supplemented with 4% (vol/vol) horse

serum and 5% (vol/vol) spent MMvM broth yeast culture filtrate from 2-week-old cultures.

Peritoneal macrophages. Peritoneal cells (PC) were collected from the abdominal cavity of each mouse by repeated lavage with 10 ml of minimal essential medium containing heparin (10 U/ml). PC from five or more mice were pelleted by centrifugation (200 × *g*, 10 min) and pooled. PC were dispensed (0.1 ml of 5 × 10^6 PC/ml of CTCM) into wells of 96-well (Costar 3696; Costar Corp., Cambridge, Mass.) microtest plates. The PC were incubated at 37°C in 5% CO_2–95% air for 2 h, and then nonadherent cells were aspirated and each well was washed once with CTCM. The number of nonadherent cells was subtracted from the number of incubated PC. The average number of adherent cells per well was 2.5 × 10^5. For microscopic studies of intracellular multiplication, PC (0.25 ml of 2.5 × 10^6 PC/ml of CTCM) were dispensed into wells of eight-chambered Lab-Tek tissue culture slides (Miles Scientific, Naperville, Ill.). Macrophage monolayers were obtained as described above and yielded an average of 0.3 × 10^6 macrophages per well.

Pulmonary macrophages. Lungs of mice were lavaged in situ with Dulbecco phosphate-buffered saline without calcium or magnesium containing 0.1% EDTA as previously described (15). Lavaged cells were pelleted by centrifugation (200 × *g*, 10 min) and pooled. Pooled cells were washed once with CTCM, counted with a hemacytometer, and suspended at a density of 2.5 × 10^6 cells/ml of CTCM. Macrophage monolayers were formed by incubating 0.1 ml of cell suspension per well of Costar 3696 microtest plate at 37°C in 5% CO_2–95% air for 2 h. After incubation, nonadherent cells were aspirated and the monolayers were rinsed once with CTCM. Approximately 90% of incubated cells adhered, and monolayers contained an average of 2.2 × 10^5 macrophages per well.

Spleen cell culture supernatants. Spleen cells were obtained from mice as previously described (4, 10). Briefly, 5 × 10^6 spleen cells per ml of CTCM were incubated (37°C in 5% CO_2–95% air) with or without ConA (5 μg/ml) for 24 h in 24-well tissue culture plates (2 ml per well). Cell-free supernatants were obtained by passage of culture supernatants through 0.45-μm-pore filters (Millipore Corp., Bedford, Mass.). Portions of supernatants were stored at −70°C until needed.

Lymph node cells. In some studies, lymph node cells were used as a control for macrophages. These cells were obtained as described previously (6).

Treatment of macrophage monolayers. Macrophage monolayers in microtest plate wells were treated overnight at 37°C in 5% CO_2–95% air with 0.1 ml of CTCM, CTCM containing interferon (100 or 1,000 U/ml), or 0.1 ml of undiluted supernatants from ConA-stimulated spleen cells containing 50 mM α-methyl-D-mannoside to neutralize ConA (4). For treatment of macrophage monolayers in Lab-Tek cultures, 0.25-ml volumes of the above reagents were used.

Challenge of macrophages and determination of CFU. After treatment of macrophages, culture supernatants were aspirated and monolayers challenged with 0.1 ml of *P. brasiliensis* yeast cells (10,000 viable units per ml of CTCM containing 10% fresh mouse serum). Viability of the *P. brasiliensis* inoculum was determined by fluorescein diacetate-ethidium bromide staining, and the morphology was determined microscopically to be primarily single-cell units (90%) and some mother cells with four or more buds (5%). After incubation of cocultures at 37°C in 5% CO_2–95% air for 4, 24, 72 or 144 h they were harvested by aspiration with distilled water to lyse macrophages. Each culture and its well washings were contained in a final volume of 10 ml. The number of CFU per culture was determined by plating 1 ml of the 10-ml harvest volume per BHI–4% horse serum–5% yeast culture filtrate agar plate. Colonies per plate were counted after 8 to 10 days of incubation at 35°C. After the first day of incubation, plates were incubated in plastic bags to prevent dessication.

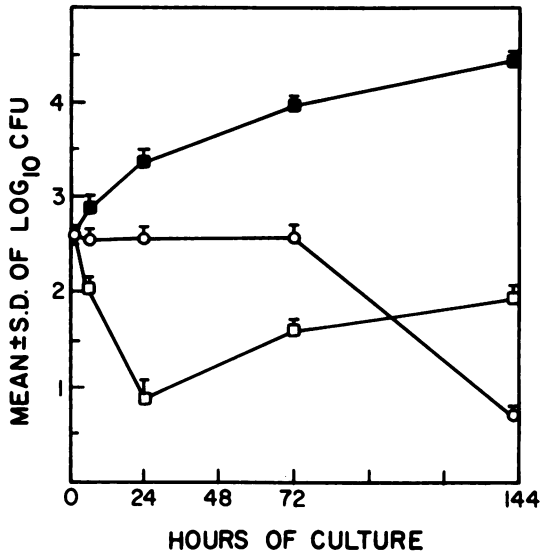

FIG. 1. Multiplication of *P. brasiliensis* (Gar APw) in peritoneal macrophage cocultures. Mean ± standard deviation (bars) of $\log_{10}$ CFU from quadruplicate cocultures of *P. brasiliensis* with nonactivated macrophages (■), with activated macrophages (□), or with CTCM (○) at 0, 24, 72, and 144 h are shown.

Microscopic determination of intracellular multiplication. Peritoneal macrophages and *P. brasiliensis* (ratio of 5:1) were cocultured in chambers of Lab-Tek slides at 37°C in 5% CO_2–95% air for 4, 24, 48, or 72 h. After incubation, quadruplicate sets of cultures were aspirated, washed three times with phosphate-buffered saline (0.25 ml per well), air dried, and stained with Diff-Quick stain (American Scientific Products, McGaw Park, Ill.). One hundred macrophages that had ingested *P. brasiliensis* were examined per culture, and the morphology of *P. brasiliensis* was recorded. A single cell or a budding cell (including multiply budding cells) was counted as one fungal unit, and the number of cells per unit was recorded. The mean ± standard deviation of quadruplicate cultures was determined and used for statistical analysis.

Statistics. Comparisons between groups were analyzed by the Student's *t* test, with significance assumed to be $P < 0.05$.

RESULTS

Effect of peritoneal macrophages on *P. brasiliensis* multiplication. When resident peritoneal macrophages were cocultured with *P. brasiliensis* Gar APw, multiplication (as determined by CFU) increased severalfold compared with *P. brasiliensis* in CTCM alone (Fig. 1). Inoculum CFU (385 ± 42) increased 5.7 times in 24 h (2,210 ± 550 CFU), 21.0 times in 72 h (8,110 ± 550 CFU) and 70.5 times (27,160 ± 4,090 CFU) in 144 h in the presence of resident peritoneal macrophages ($P < 0.001$ for all three time periods compared with inoculum). *P. brasiliensis* did not multiply in CTCM in 72 h, and with time (144 h) became nonviable (Fig. 1).

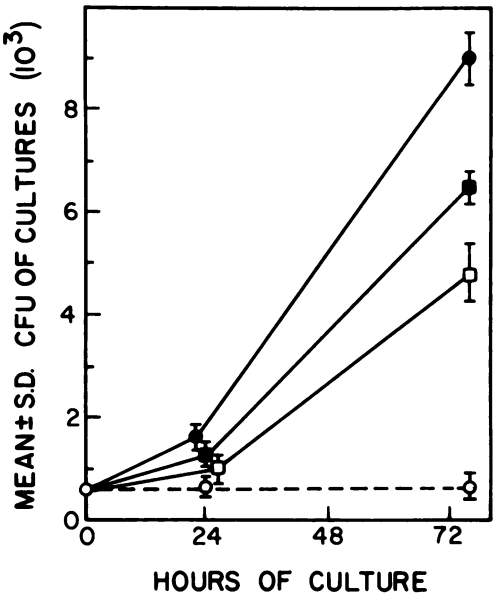

FIG. 2. Effect of nonactivated peritoneal macrophage density on growth of *P. brasiliensis* (Gar APw). Mean CFU ± standard deviation (bars) of quadruplicate cocultures of *P. brasiliensis* and 2.5×10^5 (●), 1.2×10^5 (■), or 0.6×10^5 (□) nonactivated macrophages at 0, 24, and 72 h are shown. CFU of *P. brasiliensis* in CTCM (○) is also shown.

Enhancement of *P. brasiliensis* multiplication increased with the increased number of resident peritoneal macrophages in the coculture (Fig. 2). The inoculum CFU (520 ± 62) increased 9.3 times (4,875 ± 687 CFU), 12.4 times (6,470 ± 418 CFU), and 17.6 times (9,170 ± 720 CFU) when cocultured with approximately 0.6×10^5, 1.2×10^5, or 2.5×10^5 macrophages, respectively, for 72 h (Fig. 2). The increase in CFU with each increase of macrophages was significant ($P < 0.01$ [comparison of each increment in macrophages with the next lower concentration]), and the increase in fungal cells over 72 h in all macrophage cocultures was highly significant ($P < 0.001$) compared with the inoculum.

Increases in CFU harvested from cocultures could not be attributed to a significant shift in multicellular units to single-cell units with time in culture. The percentage of single-cell units was 86, 88, and 75 when culture material harvested at 24, 72, and 144 h was examined microscopically ($P > 0.05$).

In sharp contrast to multiplication of *P. brasiliensis* in resident peritoneal macrophage cocultures, the viability of *P. brasiliensis* was rapidly reduced in the presence of lymphokine-activated macrophages (Fig. 1). Inoculum CFU (385 ± 42) were reduced 64% (140 ± 27) in 4 h ($P < 0.001$) and by 98% (7.5 ± 9.0) in 24 h ($P < 0.001$). *P. brasiliensis* that survived after 24 h increased to 97 ± 23 CFU with 120 h of additional coculture time (Fig. 1).

Multiplication of other *P. brasiliensis* isolates in macrophage cocultures. Two other isolates of *P. brasiliensis*, ATCC 32074 (LA) and ATCC 60855 (Gra), were cocultured with nonactivated and lymphokine-activated peritoneal macrophages. Inoculum CFU (373 ± 20 of ATCC 32074 and 743 ± 56 CFU of ATCC 60855) increased 13.5- and 7.4-fold, respectively ($P < 0.001$) in 72-h cocultures (Table 1). Viability of these isolates, in contrast to Gar APw, decreased by 85 and 70%, respectively, in 72-h CTCM cultures (Table 1).

TABLE 1. Multiplication of *P. brasiliensis* isolates in peritoneal macrophage cocultures

Culture	Multiplication of *P. brasiliensis* isolate:		
	Gar APw[a]	ATCC 32074[b]	ATCC 60855[b]
CTCM	0.92 ± 0.06	0.15	0.30
Nonactivated macrophages[c]	19.30 ± 2.40	13.50	7.42
Activated macrophages[d]	0.04 ± 0.05	0.01	0.02

[a] Multiplication index: (CFU at 72 h)/(CFU at 0 h). Mean ± standard deviation in two experiments.
[b] Multiplication index: mean of quadruplicate cultures in a single experiment.
[c] CTCM-treated peritoneal macrophages (nonactivated).
[d] Peritoneal macrophages treated overnight with ConA supernatants.

In 144 h, ATCC 32074 and ATCC 60855 cocultured with macrophages multiplied 22.1- and 23.8-fold, respectively (data not shown). On the other hand, it is shown in Table 1 that lymphokine-activated peritoneal macrophages reduced inoculum CFU of Gar APw (96%), ATCC 32074 (99%), and ATCC 60855 (98%) in 72 h ($P < 0.001$ for all three isolates).

Effect of pulmonary macrophages on *P. brasiliensis* multiplication. Incubation of resident pulmonary macrophages with *P. brasiliensis* resulted in multiplication of *P. brasiliensis* 5.3-fold in 144-h cocultures (Fig. 3). Inoculum CFU (145 ± 23) increased to 772 ± 56 CFU in 144 h ($P < 0.001$) in the presence of pulmonary macrophages. Coculture with an equal number of lymph node cells did not have this effect; however, the presence of lymph node cells enabled *P. brasiliensis* to remain viable during the 144-h culture period (Fig. 3). This may have been due to the presence of some macrophages in lymph node cell preparations. By contrast, CTCM alone could not maintain the viability of *P. brasiliensis* in 144-h cultures. On the other hand, lymphokine-acti-

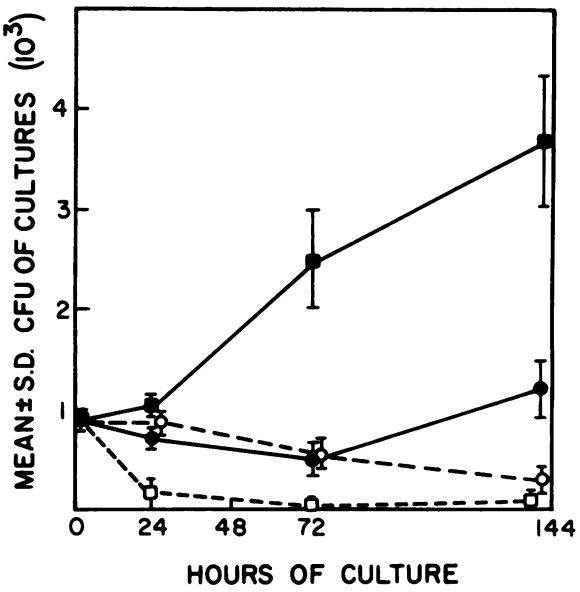

FIG. 3. Multiplication of *P. brasiliensis* (Gar APw) in pulmonary macrophage cocultures. Mean ± standard deviation of CFU from quadruplicate cocultures of *P. brasiliensis* and nonactivated macrophages (■), activated macrophages (□), or lymph node cells (●) at 0, 24, 72 and 144 h are shown. Mean ± standard deviation of CFU of *P. brasiliensis* in CTCM (○) at these times is also shown.

TABLE 2. Intracellular multiplication of *P. brasiliensis* ingested by peritoneal macrophages

Culture type and time (h)	% of macrophages with the following no. of fungal cells/unit[a]:				
	1	2	3	5	≥5
Nonactivated macrophages					
4	92.7 ± 1.5	1.2 ± 0.9	1.0 ± 0.0	0.0 ± 0.0	4.7 ± 0.5
24	61.0 ± 2.9	1.5 ± 0.5	0.7 ± 0.9	0.5 ± 0.5	36.2 ± 2.9
48	10.2 ± 5.3	2.2 ± 2.6	4.0 ± 3.1	3.7 ± 1.7	79.5 ± 6.6
Activated macrophages[b]					
4	92.2 ± 1.5	1.2 ± 1.2	1.2 ± 0.9	0.5 ± 0.5	4.7 ± 0.5
24	93.5 ± 2.8	0.7 ± 1.5	0.7 ± 0.5	1.2 ± 0.5	3.7 ± 1.8
48	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Percentage of macrophages with ingested fungal unit consisting of one, two, etc. *P. brasiliensis* cells (Gar APw) per fungal unit (Diff-Quick staining) ingested by macrophages; mean ± standard deviation of quadruplicate determinations, 100 macrophages per determination.
[b] Macrophages treated overnight with ConA supernatants.

vated pulmonary macrophages reduced inoculum CFU of *P. brasiliensis* by 81 ± 14% in 24-h ($P < 0.001$) and by 100 ± 0% in 72-h ($P < 0.001$) cocultures (two experiments) (Fig. 3).

Microscopic determination of intracellular multiplication of *P. brasiliensis*. Resident peritoneal macrophages were treated overnight with CTCM or supernatants from ConA-stimulated spleen cell cultures in chambers of Lab-Tek tissue culture slides. Treated macrophages were challenged with *P. brasiliensis* in a macrophage-to-fungus ratio of 5:1, and the morphology of ingested *P. brasiliensis* was monitored microscopically with time. At 4 h after challenge, the morphology of *P. brasiliensis* ingested by nonactivated and activated macrophages was similar, e.g., 92.7 versus 92.2% with single-cell units and 4.7 versus 4.7% with ≥5 cells per unit (Table 2). After 4 h, *P. brasiliensis* ingested by nonactivated macrophages stained uniformly dark purple, whereas there was variable staining of *P. brasiliensis* in activated macrophages (i.e., some cells had speckled staining and others were ghosts) (Fig. 4A).

After 24 h of coculture, intracellular multiplication of *P. brasiliensis* in nonactivated macrophages was evident by the increased numbers of macrophages with budding mother cells (Table 2 and Fig. 4B). Multiplication in nonactivated macrophages continued with time, so that in 48 h 79.5% of the macrophages contained budding mother cells with four or more buds (Table 2 and Fig. 4C). It was not possible to evaluate the 72-h nonactivated macrophage cocultures because of the deterioration of macrophages and extensive growth of *P. brasiliensis*.

In sharp contrast to these results, *P. brasiliensis* ingested by activated macrophages failed to multiply; i.e., the percentage of macrophages with multicellular fungal units did not increase in 24 h (Table 2). In 48 h, *P. brasiliensis* cells ingested by activated macrophages were completely digested and eliminated (Table 2) (Fig. 4D).

Growth of *P. brasiliensis* in media supplemented with culture filtrate, macrophage supernatants, or lysates. In the microtest plate well culture system at 37°C in 5% CO_2–95% air, *P. brasiliensis* Gar APw did not multiply in CTCM in 72 h (multiplication index, 0.92 ± 53) and started to die in 144 h (multiplication index, 0.18 ± 0.25) (Table 3). When *P. brasiliensis* yeast-form 2-week MMvM culture filtrate (5% [vol/vol]) was added to CTCM, multiplication of Gar APw was not improved in this system (Table 3). When Gar APw was cultured in MMvM or MMvM plus culture filtrate, multiplication was not significantly enhanced, but survival over 144 h was (Table 3). When Gar APw was cultured in supernatants from 72-h peritoneal macrophage cultures it did not multiply, but it did survive for 144 h (Table 3). Macrophage lysates combined with CTCM also did not promote multiplication of Gar APw in 72 h (Table 3). Taken together, these results indicate that these media or supplemented media under these culture conditions were not supportive of multiplication of *P. brasiliensis*.

DISCUSSION

In previous work with short-term 4-h assays, we reported that peritoneal and pulmonary macrophages readily phagocytosed *P. brasiliensis* yeast cells (2, 3) and that only activated macrophages were fungicidal. In the present report we have extended these studies by doing time course investigations and microscopic observations. We found that *P. brasiliensis* yeast cells (90% single cells) were ingested by nonactivated macrophages and that they multiplied several-fold inside macrophages. These microscopic observations were verified and quantitated by determination of CFU per culture by plating. This is the first demonstration that *P. brasiliensis* can grow intracellularly, and this new finding has important implications relevant to the pathogenesis of *P. brasiliensis*.

Although numerous histopathological studies have described host-parasite interactions in experimental paracoccidioidomycosis (5, 8, 9), it was not possible to determine by these methods precisely where *P. brasiliensis* replicated. Our in vitro results suggest that in vivo it is likely that *P. brasiliensis* multiplies intracellularly after ingestion by nonactivated macrophages and that this is followed by destruction of macrophages and release of numerous yeast cells. The possibility of extracellular multiplication of *P. brasiliensis* in vivo has not been ruled out by our in vitro results. However, environments well suited for mammalian cell growth, e.g., CTCM, failed to support multiplication of *P. brasiliensis* even when supplemented with cellular products or debris.

A rate-limiting factor in the multiplication of *P. brasiliensis* in vitro is the presence of siderophores for iron transport (1, 7a). However, *P. brasiliensis* did not multiply in CTCM or MMvM when siderophores (present in spent culture medium filtrate) were added. Apparently these culture conditions, 37°C and 5% CO_2–95% air, are not favorable for multiplication of *P. brasiliensis*. We speculate that macrophages provide an environment and transport mechanism for ingested *P. brasiliensis* and thereby support *P. brasiliensis* multiplication.

In dramatic contrast to nonactivated macrophages, gamma interferon- or lymphokine-activated macrophages not only prevented multiplication of ingested *P. brasiliensis*

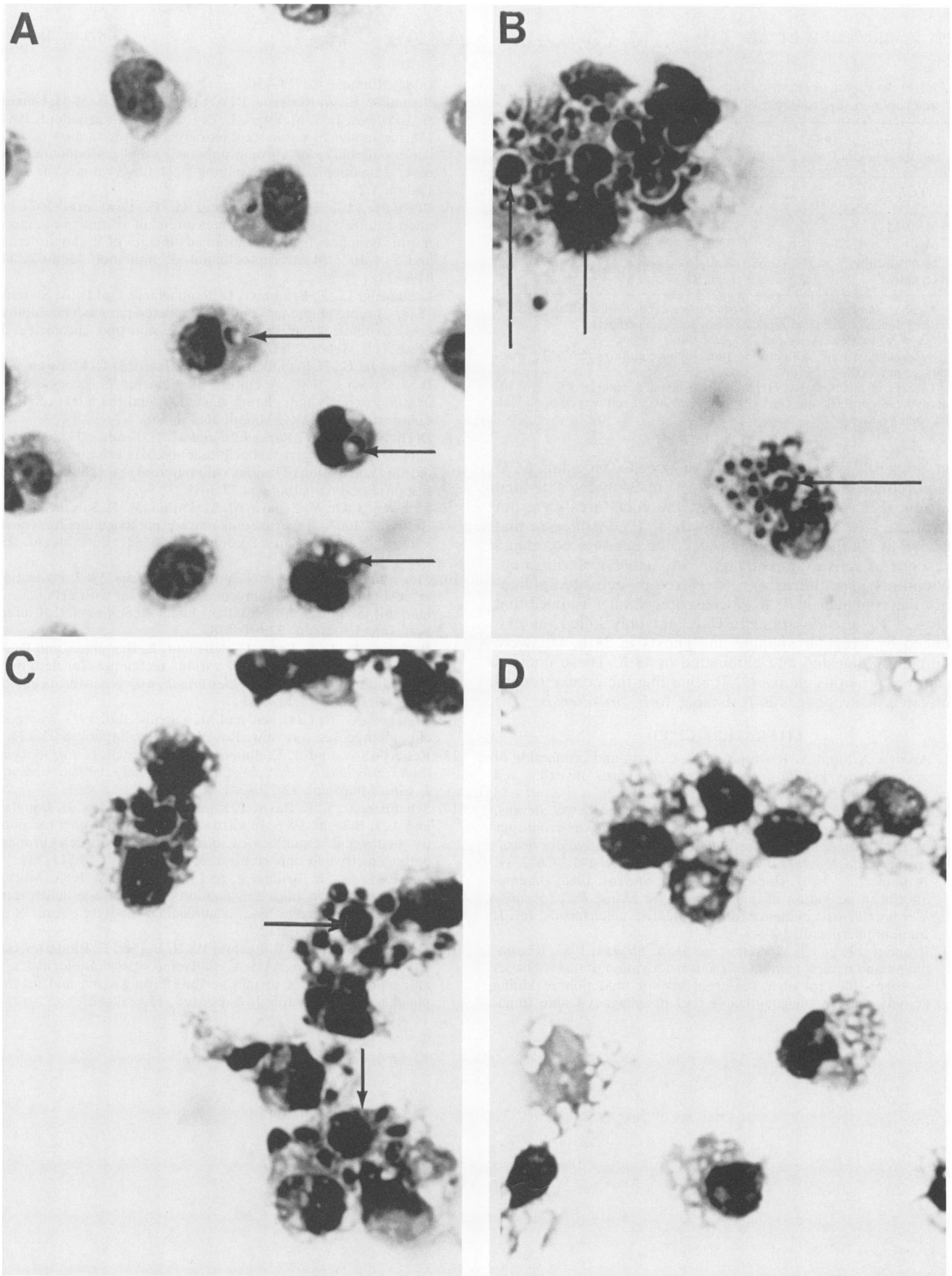

FIG. 4. Intracellular multiplication of *P. brasiliensis* (Gar APw) in macrophages. (A) Single yeast cells (arrows) ingested by activated peritoneal macrophages. (B) Multiplication of *P. brasiliensis* in nonactivated peritoneal macrophages in 24 h (arrows indicate mother cells with buds). (C) Multiplication of *P. brasiliensis* in nonactivated peritoneal macrophages in 48 h (arrows indicate mother cells with buds). (D) Destruction of yeast cells in activated macrophages at same time as shown in panel C.

TABLE 3. Effect of spent culture filtrate, macrophage lysates, or macrophage supernatants on multiplication of *P. brasiliensis*

P. brasiliensis Gar APw culture conditions	Multiplication index (mean ± SD) after[a]:	
	72 h	144 h
CTCM	0.92 ± 0.53	0.18 ± 0.25
CTCM + CF[b]	0.96 ± 0.01	0.33
MMvM	1.16 ± 0.19	1.09
MMvM + CF[b]	0.84	1.48
Supernatants[c]	0.78	0.93
Lysates[d]	0.87	

[a] Mean ± standard deviation of two or more experiments. Means without standard deviation are from replicates in a single experiment.
[b] Spent MMvM culture filtrate, 5% (vol/vol).
[c] Supernatants from 72-h peritoneal macrophage cultures (2.5×10^6 macrophages per well).
[d] Lysates of macrophages prepared using distilled water at 4°C, 2×10^5 macrophages per 0.025 ml. Gar APw in CTCM (0.075 ml) was cultured with 0.025 ml of lysate.

but efficiently killed and digested yeast cells. Inoculum CFU were reduced by greater than 85% in 24 h by activated peritoneal macrophages; however, the cocultures were not sterilized. The survival and growth of *P. brasiliensis* that survive the initial killing may reflect the need for continued presence of activating lymphokines to achieve sterilization. By contrast, cocultures were sterilized by activated pulmonary macrophages in 72 h. Microscopic studies showed that ingested *P. brasiliensis* cells were not only killed, as evidenced by irregular staining and ghosts in 24 h, but were completely digested and eliminated in 48 h. These findings reinforce previous claims (2, 3) regarding the crucial role of activated macrophages in resistance to *P. brasiliensis*.

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