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Development of an In Vitro Macrophage System To Assess *Penicillium marneffei* Growth and Susceptibility to Nitric Oxide

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We investigated the effect of nitric oxide (NO) and reactive nitrogen intermediates on the in vitro growth of *Penicillium marneffei* both in a cell-free system and in a novel macrophage culture system. In the cell-free system, NO that was chemically generated from $NaNO_2$ in acid media (pH 4 and 5) markedly inhibited the growth of *P. marneffei*. On the contrary, inhibition of growth did not occur in neutral medium (pH 7.4) in which NO was not produced. *P. marneffei* conidia were phagocytized by nonstimulated murine J774 macrophages after 2 h of incubation. During the following 24 h, *P. marneffei* grew as yeast-like cells replicating by fission in the J774 macrophages. The intracellular growth of *P. marneffei* damaged nonstimulated J774 macrophages, as confirmed by electron microscopy. When J774 cells were stimulated by gamma interferon and lipopolysaccharide, which led to enhanced production of reactive nitrogen intermediates, the percentage of yeast-like cells was significantly reduced and *P. marneffei* conidia were damaged in the J774 macrophages. The inhibition of NO synthesis by *N*-monomethyl-L-arginine restored the intracellular growth of *P. marneffei*. The inverse correlation between intramacrophage growth and the amount of nitrite detected in culture supernatants supports the hypothesis that the L-arginine-dependent NO pathway plays an important role in the murine macrophage immune response against *P. marneffei*.

Penicillium marneffei is a dimorphic fungus endemic in Southeast Asia. Occasionally described as the cause of deep-seated infection in the pre-AIDS era, this fungus is now considered an emerging pathogen in human immunodeficiency virus (HIV)-positive patients living or travelling in Southeast Asia, where the AIDS epidemic is burgeoning (6, 21). In Northern Thailand, disseminated penicilliosis marneffei is the third most common opportunistic infection in HIV-positive individuals after extrapulmonary tuberculosis and cryptococcal meningitis, and the disease was recently designated an indicator of AIDS by the Thai Ministry of Public Health (10).

The routes and mechanisms of infection by *P. marneffei* as well as the host immune response are still poorly understood. Phagocytic cells are likely to represent one of the primary lines of defense against it.

Nitric oxide (NO) has recently been studied as an antimicrobial factor produced by murine and to a lesser extent by human macrophages (16, 20). Expression of inducible NO synthase enables macrophages to increase the production of NO from L-arginine and oxygen. The increased concentration of NO and reactive nitrogen intermediates (RNI) produced by activated macrophages is reported to inhibit the growth of several bacteria, protozoa, fungi, and viruses (4).

Immune-stimulated murine macrophages were recently reported to inhibit the growth of *Candida albicans* (2), *Cryptococcus neoformans* (9), *Histoplasma capsulatum* (14, 17), *Pneumocystis carinii* (11), and *Rhizopus* spp. (12) by RNI production. However, no reports have been published on the po-

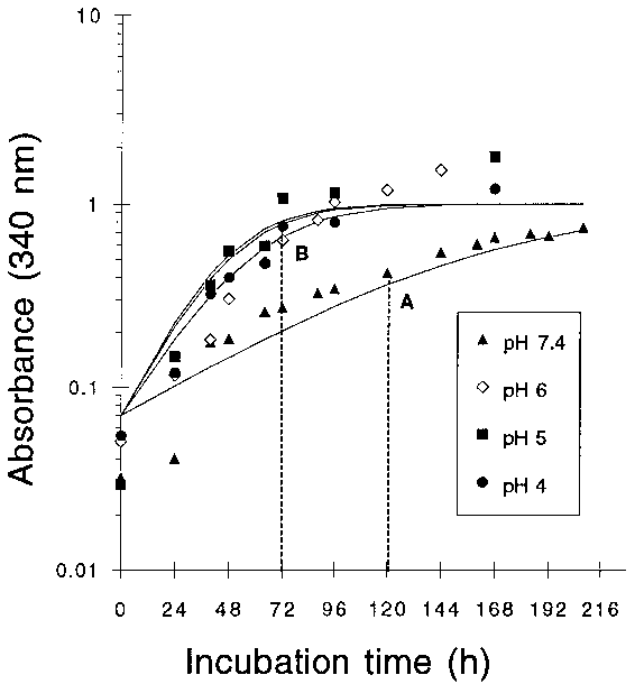

FIG. 1. *P. marneffei* growth curve at 37°C in YNBGA medium at different pHs. Each point is the mean for triplicate determinations. Optimal incubation times for spectrophotometrical measurements are indicated by A (neutral medium) and B (acidic media).

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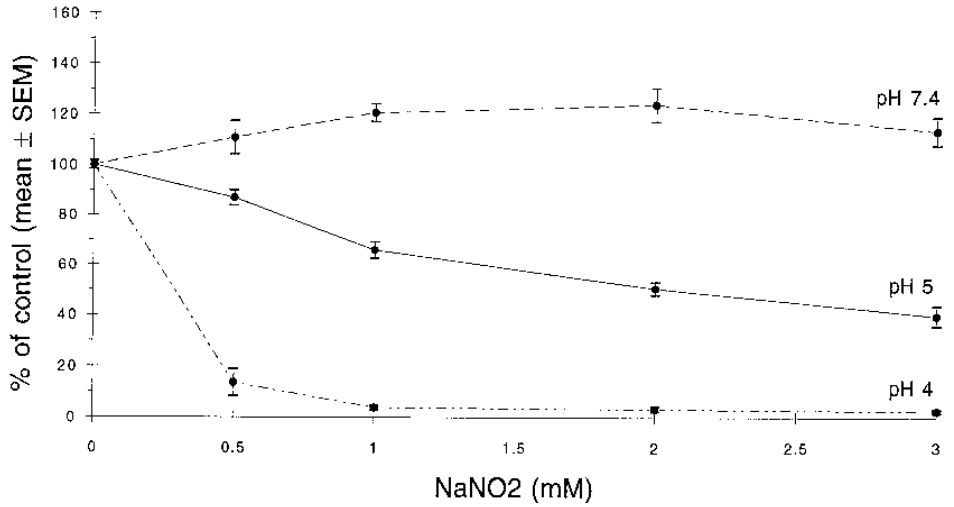

FIG. 2. Effect of NO, chemically generated from $NaNO_2$ in axenic medium at pH 4, 5, and 7.4, on *P. marneffei* growth. Cultures were inoculated with 10^6 conidia per ml and incubated at 37°C for 72 h (acid media) or 120 h (neutral medium). Growth was evaluated by spectrophotometry at 340 nm. Each point is the mean ± SEM of two independent experiments performed in triplicate. Control growth is equal to 100%.

tential effect of NOs on *P. marneffei*. We designed a study to evaluate the effect of NO and RNI, generated either chemically or after immunological stimulation of murine macrophages, on the in vitro growth of *P. marneffei*. The in vitro model that we developed is the first one to reproduce the transformation of the fungus from a conidial to a yeast-like form, the latter form being found in vivo in infected macrophages.

MATERIALS AND METHODS

Strain. *P. marneffei* IUM 885346 was used in this study. It was isolated from the blood of an HIV-positive, intravenous-drug-addicted Italian male who had travelled in Thailand (23). The fungus was maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) at 25°C.

Inoculum preparation. *P. marneffei* conidia, obtained from a culture on Sabouraud dextrose agar grown at 25°C for 10 to 14 days, were collected by flooding the culture surface with sterile distilled water. A total of 10^5 conidia were inoculated on yeast morphology agar (Difco) and incubated at 25°C. After 8 days, conidia were collected as described above and the suspension was filtered through a glass wool column. The eluate containing mycelium-free conidia was centrifuged for 10 min at 1,800 × *g*. The pellet was washed twice in sterile distilled water and then diluted to a final suspension of 10^7 conidia per ml, as estimated with a Bürker hemocytometer.

Determination of the growth curve in an axenic medium. (i) Medium and culture conditions. The growth curve of *P. marneffei* was determined in the same medium at four different pH values (4, 5, 6, and 7.4). The medium was composed of 0.17% yeast nitrogen base without amino acids and without ammonium sulfate (Difco), 1% glucose, 0.1% asparagine, and a 2% RPMI 1640 amino acid solution (Sigma-Aldrich, Milan, Italy), which were dissolved in different buffering solutions (YNBGA medium). Succinic acid at 25 mM was used to prepare the media at pH 4 and 5. The pH was adjusted by adding 1 N NaOH. The medium was filter sterilized (0.22-μm pore size; BioDisc; C.A.M. Bio, Milan, Italy). Sodium phosphate buffer (NaH_2PO_4 and Na_2HPO_4) was used to prepare the media at pH 6 and 7.4. Media were dispensed in vials and inoculated with *P. marneffei* (10^6 conidia per ml). Triplicate cultures were incubated at 37°C.

(ii) Spectrophotometric estimation of growth. At 24-h intervals, one culture from each pH series was sonicated at 50 W for 3 min with an ultrasonic homogenizer (Labsonic 2000; Braun). Each suspension was transferred to a 10-mm-light-path polystyrene cuvette (capacity, 2 to 4 ml), and A_{340} was determined (Beckman model DU 70 spectrophotometer). Background absorbance due to the medium was subtracted. Incubation times used in the following experiments were chosen within the exponential-phase interval.

Culture in NO-generating media. Three media with different pHs (4, 5, and 7.4) were prepared as described above. Each medium was distributed in vials, and $NaNO_2$ was added at different concentrations (0.5, 1, 2, and 3 mM). Control vials contained only the medium. Triplicate cultures were inoculated with 10^6 *P. marneffei* conidia per ml and were incubated at 37°C. After the incubation time, which was chosen as described above, cultures were sonicated and growth was measured spectrophotometrically.

Macrophage cell line. A murine macrophage-like cell line (J774) was maintained in culture dishes containing 10 ml of minimal essential medium (GIBCO-BRL, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Rehatuin; Intergen), 1% glutamine (GIBCO-BRL), 1% nonessential amino acid solution (GIBCO-BRL), 2% Tricine (GIBCO-BRL), and a 1% penicillin-streptomycin solution (GIBCO-BRL) and was incubated at 37°C in 5% CO_2. J774 macrophages were collected with a cell scraper (Cell Lifter; Costar Italia, Milan, Italy) and transferred to fresh medium every 3 days.

Macrophage assaying. J774 macrophages (10^5 per well) were seeded into a four-well chamber slide (Nunc, Roskilde, Denmark) containing minimal essential medium prepared as described above and were incubated at 37°C in 5% CO_2. After 20 h, confluent monolayers were obtained and treated with *P. marneffei* conidia ($2 \cdot 10^5$ per well) in the presence or absence of 10 U of recombinant murine gamma interferon (rMuIFN-γ; Genzyme, Cambridge, Mass.) and 1 μg of lipopolysaccharide (LPS [O111:B4]; Sigma) per ml. In some experiments, rMuIFN-γ- and LPS-stimulated J774 cells were treated with 250 μM *N*-monomethyl-L-arginine (LNMMA; Calbiochem, San Diego, Calif.) to inhibit NO synthesis. Phagocytosis was allowed for 2 h in the absence of opsonins, and J774 monolayers were washed with warm phosphate-buffered saline (PBS) solution to remove nonphagocytized conidia and were then treated again with stimuli as described above for a further 24 h. Finally, supernatants from each well were collected and assayed for the presence of nitrite. The slides were then fixed and stained.

Nitrite assaying. The accumulation of nitrite was measured by mixing 100 μl of culture supernatants with an equal volume of Griess reagent (1% sulfanilamide–0.1% naphthylethylenediamine dihydrochloride in 5% concentrated H_3PO_4). Plates were incubated at room temperature for 10 min, and then A_{550} was measured in a microtiter plate reader (Molecular Devices). The nitrite concentrations (in nanomoles per sample) were determined by a least-squares linear regression analysis of a sodium nitrite standard curve (from 5 to 100 μM)

TABLE 1. Correlation between intracellular growth of *P. marneffei* and nitrite produced by J774 macrophages

Row	rMuIFN-γ (10 U/ml)	LPS (1 μg/ml)	LNMMA (250 μM)	% Yeast-like cells[a]	Amt of NO_2^- (nmol/well)[b]
1	−	−	−	94.2	1.2 ± 0.08
2	+	+	−	11.3[c]	22.9 ± 0.08[c]
3	+	+	+	91.2	6.3 ± 0.50[d]

[a] Intracellular growth was calculated as the percentage of yeast-like cells of the total number of fungal elements (conidia plus yeast-like cells) counted in at least 100 macrophages infected. Data are from one representative experiment repeated four times.

[b] Means ± SEM for triplicate determinations in a single experiment.

[c] $P < 0.001$ versus row 1. Statistical analysis was performed by chi-square testing (column 5) and analysis of variance (column 6).

[d] $P < 0.05$ versus row 1. Statistical analysis was as described in footnote *c*.

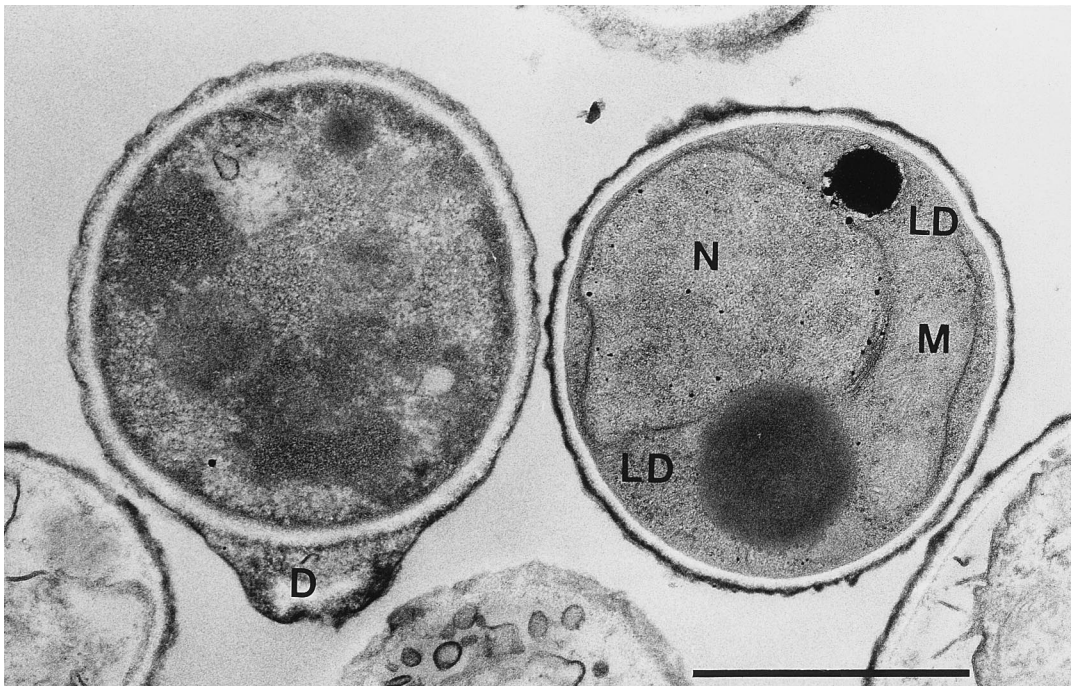

FIG. 3. *P. marneffei* conidia from the inoculum preparation. The conidium on the right has an eccentric nucleus (N), a mitochondrion (M), and two lipid droplets (LD). The conidium on the left bears a remnant of the disjunctor (D) on the thick cell wall. Bar, 1 μm.

generated for each experiment (5). Each determination was performed in triplicate.

Determination of intracellular growth. The slides were fixed with methanol for 7 min and stained with periodic acid-Schiff stain (13). Intracellular growth of *P. marneffei* was defined as the percentage of yeast-like cells observed among the total number of intracellular fungal elements (yeast-like cells plus conidia) counted in at least 100 infected macrophages.

Electron microscopy. A total of 10^6 J774 macrophages were seeded in three culture dishes containing 10 ml of minimal essential medium prepared as described above and were incubated at 37°C in 5% CO_2 for 20 h. Cells were then

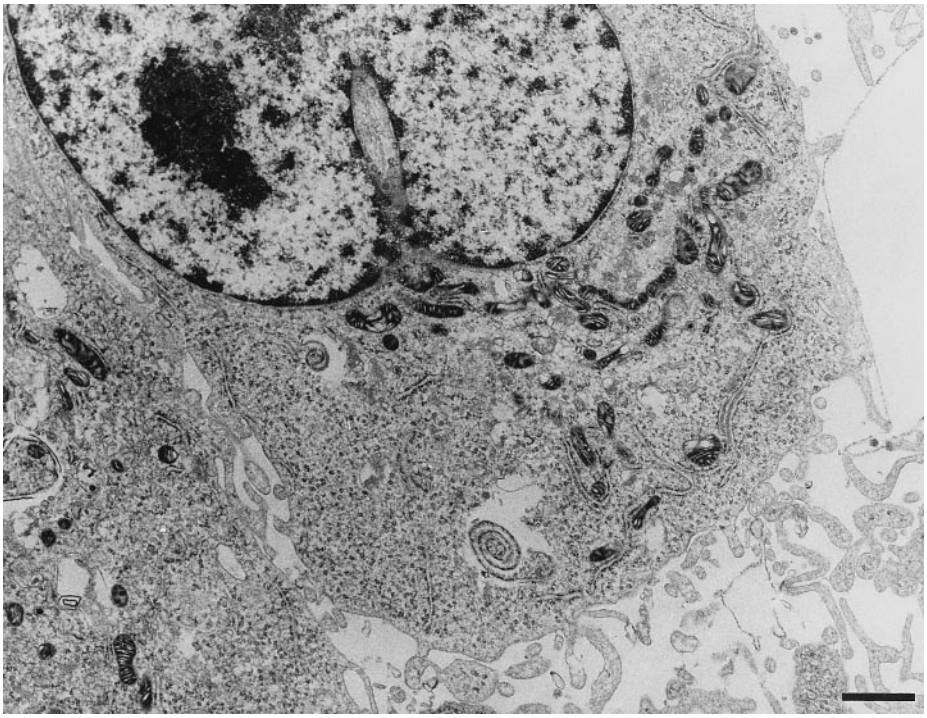

FIG. 4. J774 macrophages from a preparation not treated with conidia. Numerous cytoplasmic projections and a juxtanuclear cluster of mitochondria with a dense matrix are observed. Bar, 1 μm.

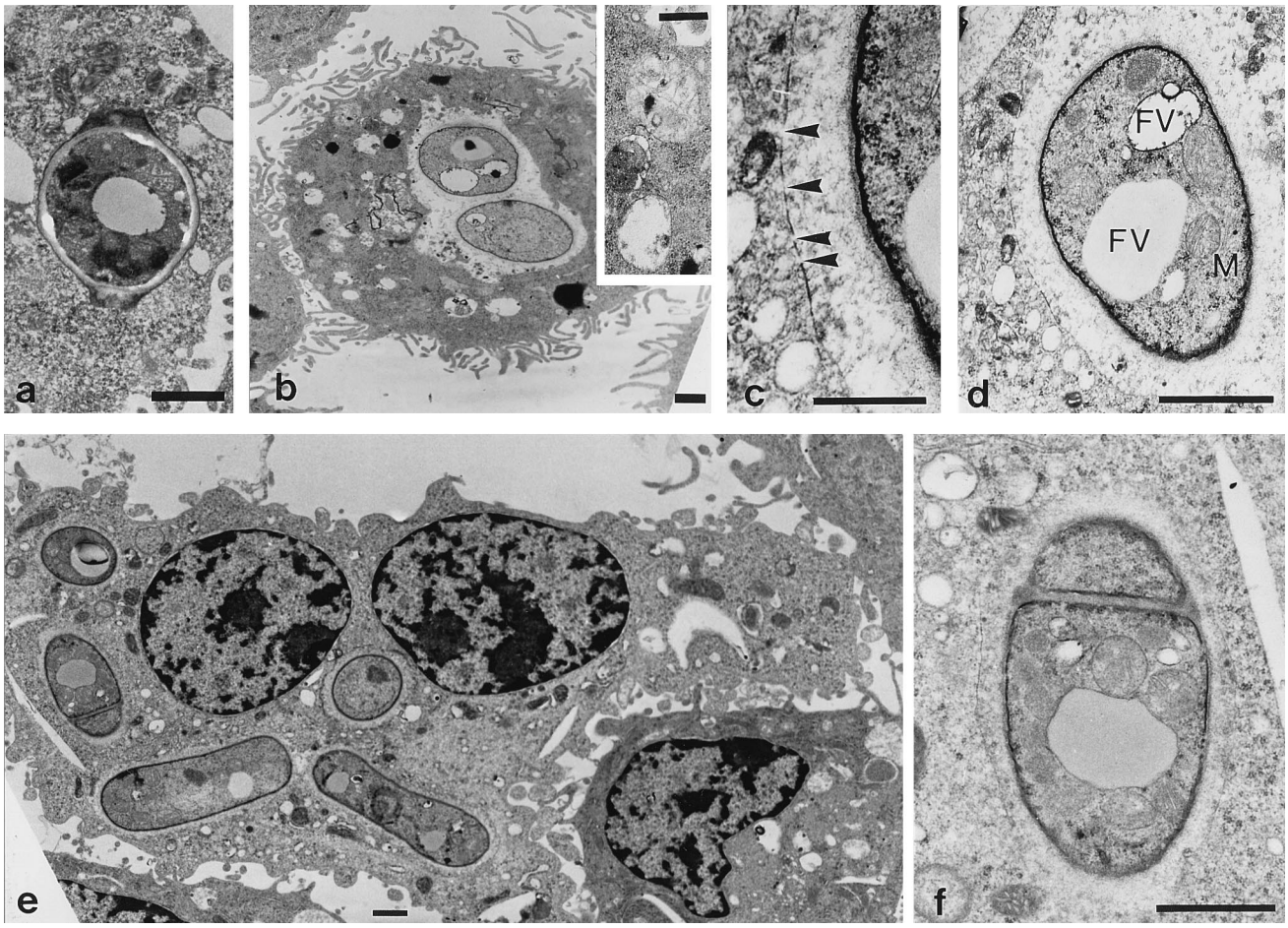

FIG. 5. Phagocytosis of *P. marneffei* by nonactivated macrophages. (a) *P. marneffei* conidium phagocytized after 2 h of incubation. Bar, 1 μm. (b) Two yeast-like cells within a large phagocytic vacuole after 24 h of incubation. Bar, 1 μm (inset, a higher magnification of two swollen mitochondria of the same cell; bar, 0.5 μm). (c) The membrane of the phagocytic vacuole, showing some discontinuities. Bar, 0.5 μm. (d) A yeast-like cell within a phagocytic vacuole showing mitochondria (M) and large food vacuoles (FV). Bar, 1 μm. (e and f) Yeast-like cells dividing by fission within phagocytic vacuoles. Bar, 1 μm.

treated with 10^7 *P. marneffei* conidia in the presence or absence of 10 U of rMuIFN-γ and 1 μg of LPS per ml and were incubated at 37°C in 5% CO_2 for another 2 h to allow phagocytosis. Subsequently, cultures were washed twice with phosphate-buffered saline. J774 cells were collected from one culture, whereas those from the others were treated with the indicated stimuli and incubated for a further 24 h before being harvested. J774 macrophages were collected with a cell scraper, transferred to a vial, and centrifuged at 3,600 rpm for 5 min. Pellets were fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 2 h, washed overnight in phosphate-buffered saline, postfixed in 2% osmium tetroxide in Millonig's buffer for 2 h, dehydrated in ethanol, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and were then examined on a transmission electron microscope (model CM 10; Philips).

Statistical analysis. Means together with standard errors of the means (SEM) are given. Statistical significance between study groups was determined by either analysis of variance or chi-square testing.

RESULTS

Influence of pH on *P. marneffei* growth. As reported in Fig. 1, the in vitro growth of *P. marneffei* in YNBGA medium at 37°C was greatly influenced by the medium pH. The fungus growth rate was enhanced in acidic compared with neutral media. Optimal incubation times for spectrophotometric measurements in acidic (pH 4, 5, and 6) and neutral (pH 7.4) media were 72 and 120 h, respectively.

Inhibition of *P. marneffei* growth in axenic NO-generating media. The effect of $NaNO_2$ on in vitro growth of *P. marneffei* in acidic and neutral media is shown in Fig. 2. $NaNO_2$ added to cultures of *P. marneffei* in YNBGA medium at pH 4 markedly inhibited fungal growth ($P < 0.01$) at all of the concentrations tested. At pH 5, growth inhibition was less pronounced but still statistically significant. In the presence of 0.5 mM $NaNO_2$, the growth was reduced to 87% of that of the control ($P < 0.05$), and in the presence of higher $NaNO_2$ concentrations, it was reduced to 66 to 39% of that of the control ($P < 0.01$). At pH 7.4, fungal growth was not reduced at any $NaNO_2$ concentrations tested. On the contrary, at 1 and 2 mM, growth increased significantly ($P < 0.01$).

Inhibition of *P. marneffei* intracellular growth by NO and RNI produced by stimulated J774 macrophages. *P. marneffei* conidia were cocultured with J774 macrophages in a 2:1 ratio for different lengths of time. After 2 h of coculture, 14.5% (SEM, ± 2.5; $n = 10$) of J774 macrophages had phagocytized a mean of 2.2 conidia per macrophage ($n = 10$). Differences in the percentages of phagocytosis in activated and nonactivated J774 macrophages were not statistically significant. In the following 24 h of culture, conidia grew as yeast-like cells and replicated by fission inside nonstimulated J774 macrophages. Periodic acid-Schiff staining of macrophage monolayers allowed conidia to be easily distinguished from yeast-like forms by light microscopy. Conidia were well-defined, roundish, and strongly stained with periodic acid-Schiff stain, whereas yeast-

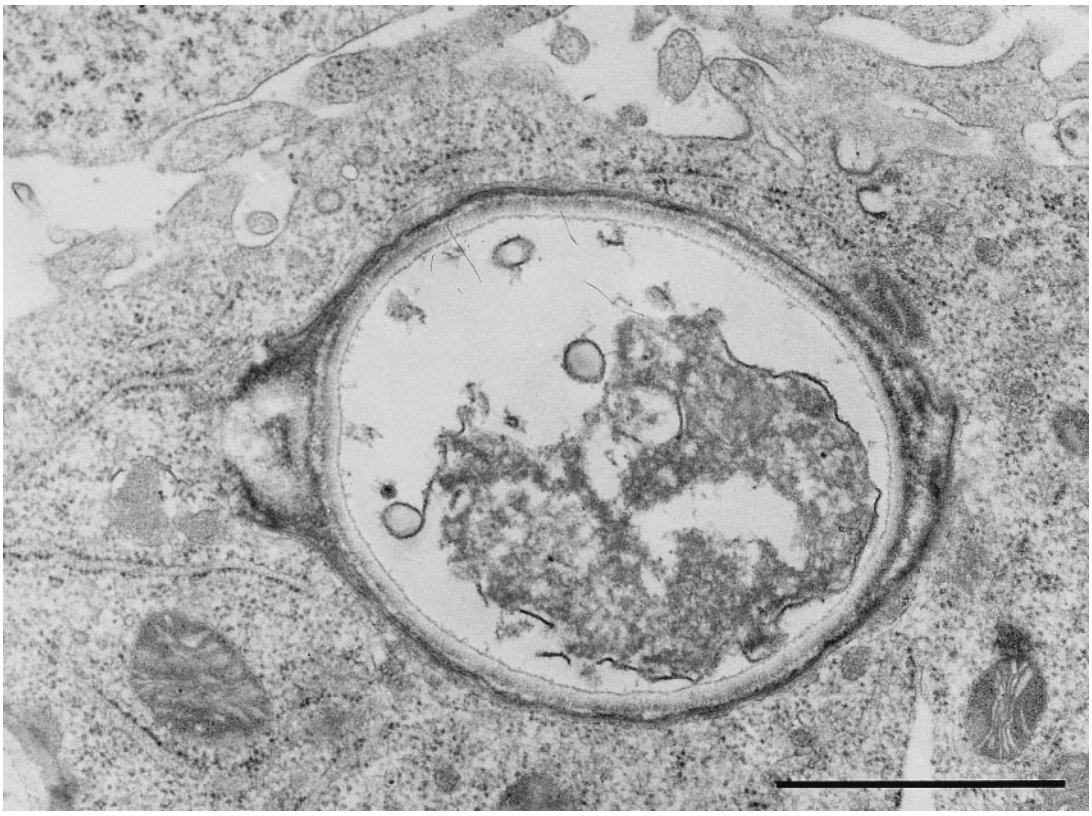

FIG. 6. A conidium of *P. marneffei* phagocytized by an activated macrophage. The fungal cytoplasm is altered, and the cell membrane appears detached from the cell wall. Bar, 1 μm.

like forms were weakly stained, swollen, and irregularly shaped. *P. marneffei* replication in J774 macrophages was confirmed by the presence of septate forms. The percentage of yeast-like cells was 94.2%, and the concentration of nitrite detected in the culture supernatant was 1.2 ± 0.08 nmol per well (Table 1, row 1). When J774 cells were stimulated by rMuIFN-γ and LPS, the percentage of yeast-like cells was significantly reduced ($P < 0.001$). The ability of activated J774 macrophages to inhibit fungal growth was directly related to the amount of nitrite (22.9 ± 0.08 nmol per well) found in the culture supernatants (Table 1, row 2).

When activated J774 macrophages were cultured in the presence of 250 μM LNMMA, NO synthesis was decreased (6.3 ± 0.5 nmol of nitrite per well) and intracellular growth of *P. marneffei* reverted to basal level (Table 1, row 3).

Electron microscopy. To confirm phagocytosis and intracellular growth of *P. marneffei* in J774 macrophages, cultures were examined by electron microscopy at different time points. Untreated J774 macrophages and conidia alone were also studied. As shown in Fig. 3, conidia from the inoculum suspension were round to oval and thick walled, with a prominent disjunctor. Nuclei with dispersed chromatin, mitochondria, and lipid droplets were evident. Untreated J774 macrophages (Fig. 4) showed numerous cytoplasmic projections and abundant mitochondria with a dense matrix.

After 2 h of incubation with J774, *P. marneffei* conidia were present in phagocytic vacuoles of nonstimulated (Fig. 5a) and activated J774 macrophages, as shown by light microscopy. The membrane of the phagocytic vacuoles was closely attached to the fungal cell wall. The ultrastructure of conidia was preserved.

When incubation was prolonged for a further 24 h, the phagocytic vacuoles of nonstimulated J774 macrophages were larger and contained yeast-like cells dispersed in a light, finely fibrillar material (Fig. 5b). J774 macrophages showed ultrastructural alterations such as mitochondrial swelling (Fig. 5b [inset]) and membrane discontinuities of phagocytic vacuoles (Fig. 5c). Yeast-like cells had an irregular shape, and the cell wall was thinner than that of conidia (Fig. 5d). Some cytoplasmic structures, such as mitochondria, rough endoplasmic reticulum, lipid droplets, and large membrane-bound food vacuoles, were evident. Yeast-like cells dividing by fission were also observed in macrophage phagocytic vacuoles (Fig. 5e to f).

The cytoplasm of J774 macrophages activated by IFN-γ and LPS showed numerous mitochondria and rough endoplasmic reticulum cisternae. Conidia in phagocytic vacuoles exhibited morphological alterations such as detachment of cell membrane from the cell wall and loss of identifiable cytoplasmic structures (Fig. 6).

DISCUSSION

The susceptibility of *P. marneffei* to NO was evaluated with a simple cell-free system in which NO is spontaneously generated by the decomposition of nitrite salts in acid aqueous solution (1). The ability of *P. marneffei* to grow in an acid medium facilitated the performance of this method (19). The addition of sodium nitrite to the acid culture media resulted in a concentration-dependent growth inhibition of *P. marneffei*. On the contrary, nitrite had no inhibitory effect on *P. marneffei* growth at pH 7.4. In this system, only two variables appeared to influence the extent of fungal growth inhibition: pH and nitrite

concentration, both of which determined the amount of NO generated in the solution.

In the present study, the J774 murine macrophage-like cell line was found to be appropriate for in vitro studies of *P. marneffei*. These cells were readily infected and supported the intracellular growth of *P. marneffei*. Previous studies have described the intracellular yeast-like form of *P. marneffei* in vivo, both in animal models and in patients with penicilliosis marneffei (3, 7, 8, 18, 22). To the best of our knowledge, our study is the first to reproduce in vitro the infectious pattern of *P. marneffei* in a cell of macrophage origin. In our model, we observed the intracellular transformation of the fungus from a conidial to a yeast-like form. Electron micrographs proved that the fungal elements were phagocytized by J774 macrophages. The increase in yeast-like cytoplasmic structures such as mitochondria, rough endoplasmic reticulum, and membrane-bound food vacuoles suggested a level of metabolic activity higher than that of resting conidia. Furthermore, findings of septate forms by both light and electron microscopy showed that *P. marneffei* replicated in J774 macrophages.

Nonactivated J774 macrophages did not inhibit the intracellular growth of *P. marneffei*, and electron micrographs revealed that J774 macrophages were damaged by the fungus, since they presented mitochondrial swelling and membrane discontinuities of phagocytic vacuoles. In contrast, immune stimulation of J774 macrophages resulted in almost complete inhibition of *P. marneffei* yeast-like transformation. The examination by electron microscopy showed that conidia phagocytized by activated macrophages were damaged. The extent of fungus damage correlated directly with the amount of nitrite found in the culture supernatants.

The addition of LNMMA to activated J774 macrophages inhibited NO production (lower nitrite levels) and restored the intracellular growth of *P. marneffei*, thus providing evidence that the L-arginine-dependent NO pathway is involved in the murine host defense against this fungus.

The inhibition of *P. marneffei* growth by NO and RNI, as demonstrated in the present study, is in agreement with results obtained for most fungi studied so far. Indeed, RNI produced by immunologically stimulated rodent macrophages have been shown to inhibit the growth of the following organisms: *C. albicans* (2), *C. neoformans* (9), *H. capsulatum* (14, 17), *P. carinii* (11), and *Rhizopus* spp. (12). However, NO does not seem to be involved in the fungicidal activity of murine alveolar macrophages against *Aspergillus fumigatus* (15).

Further studies could be carried out with this relatively simple in vitro cell system to investigate the fungus-macrophage interaction in response to immune stimulators and antifungal drugs. Studies are also needed to better understand the mechanisms by which human macrophages are able to control the growth of *P. marneffei.*

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