

Phagocytosis and Killing of Human Pathogenic, *Penicillium marneffe* and Non-pathogenic, *Penicillium citrinum* by Mouse Macrophage J774.1 Cells

Sophit Thirach, B.Sc.,
Nongnuch Vanittanakom, Dr.rer.nat.

ABSTRACT

In this study, we examined in vitro phagocytosis and killing activity of mouse macrophage cells against conidia of pathogenic *Penicillium marneffe*, and compared this with non-pathogenic *Penicillium citrinum*. Phagocytosis and killing assays were determined using microscopy and viable colony plate counts. The results indicated a highly efficient phagocytosis effect of J774.1 cells against both species of *Penicillium*. There was no difference between the percentages of cells phagocytosed in both species. The phagocytic indices of *P. marneffe* at 60, 120, and 240 minutes of infection were significantly higher than those of *P. citrinum*. However, in the early stages of phagocytosis, the percentage killing of *P. marneffe* was significantly lower than that observed in *P. citrinum*. Conidia of *P. marneffe* were more susceptible to being phagocytosed but also more resistant to killing by macrophages than those of non-pathogenic *Penicillium*. The mechanism of intracellular survival or resistance to killing by macrophages needs further investigation. (*J Infect Dis Antimicrob Agents* 2008;25:81-9.)

INTRODUCTION

Penicillium marneffe is the only thermal dimorphic pathogen among several hundred species of *Penicillium*, and is known to be pathogenic to animals and humans. It is recognized as a cause of life-threatening disseminated infection in both immunocompetent and immunocompromised hosts, particularly in patients with human immunodeficiency virus (HIV) infection. Almost all cases have been diagnosed in

patients who lived in or traveled to Vietnam, Thailand, Hong Kong, Indonesia, or southern China, where *P. marneffe* is endemic. In northern Thailand, disseminated *P. marneffe* infection is the third most common opportunistic disease in HIV-positive patients after extrapulmonary tuberculosis and cryptococcal meningitis, and the disease has been designated as an indicator of AIDS by the Thai Ministry of Public Health.¹

Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

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Reprint request: Prof. Dr. Nongnuch Vanittanakom, Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

Email: drnongnuch@gmail.com

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The exact route and mechanism of infection by *P. marneffeii* as well as the host immune response are still poorly understood. It is thought the patient could inhale conidia from a contaminated environment. This respiratory portal of entry would be consistent with infection caused by other dimorphic fungi that produce conidia in the saprophytic phase of growth. Phagocytic host cells of a host are likely to represent one of the primary lines of defense.² One study has suggested the importance of cell-mediated immunity in host resistance to *P. marneffeii* infection in a mouse model.³ Another study indicated that activated macrophages might play a role in damaging endocytosed *P. marneffeii* conidia via a nitric oxide-dependent pathway, and such a killing process might be stimulated by gamma interferon.⁴ In severe *P. marneffeii* infection, the organism proliferates in the macrophage and could disseminate throughout the body, including the reticuloendothelial system. During infection, nonspecific immunity plays a major role in the clearance of this pathogen. In this study, we examined in vitro phagocytosis and killing activity of mouse macrophage J774.1 cells against the conidia of *P. marneffeii*, compared with non-pathogenic *P. citrinum*.

MATERIALS AND METHODS

Fungal strains and media

Penicillium marneffeii CBS 119456 is a clinical isolate derived from penicilliosis marneffeii patients at Maharaj Nakorn Chiang Mai Hospital, Thailand. Non-pathogenic *Penicillium citrinum* was isolated from environment and identified by sequencing of 18S ribosomal DNA and its morphology. *P. citrinum* is not dimorphic, but it can grow at 37°C. The cultures were maintained at 28°C on Sabouraud's dextrose agar (SDA) (Becton Dickinson and Company, MD, USA). The conidia of both fungi were prepared by the following method which was modified from those described by

Cogliati and colleagues.⁴ The conidia were collected by washing and scraping the surface of colonies, which grew on potato dextrose agar (PDA) (Becton Dickinson and Company, USA) slants with a small volume of phosphate buffer saline (PBS). This was followed by filtering through sterile glass wool. The fungal conidia were collected by centrifugation at 4,500 x g for 10 min. The conidial suspension was counted with a hemocytometer; and approximately 10⁶ conidia were used immediately to infect the macrophage cells.

Macrophage culture and infection with *P. marneffeii*

The mouse macrophage J774.1 cell line (ATCC TIB-67) was cultured in Dulbecco's modified Eagle medium (DMEM) GIBCO, Invitrogen Corporation, CA, USA) supplemented with 10 percent heat-inactivated fetal bovine serum (GIBCO). Approximately 10⁵ cells were dispensed into 3 ml of DMEM medium in 12-well tissue culture plates and placed in 5 percent CO₂ at 37°C. After 3 days of macrophage cell line cultivation, the conidia were added to the macrophage monolayer (approximately 10⁵ macrophages per well) to obtain a ratio of 10 yeast cells to 1 macrophage.

Phagocytosis and killing assay

For the phagocytosis and killing assay, macrophages were cultured for 3 days in 24-well culture plates (Corning Inc., MA, USA) under the conditions described above. Each phagocytosis assay comprised 2 samples, each spiked with an identical number of conidia in a cell-free medium for the control or adherent macrophage cells for the test.⁴⁻⁵ The phagocytosis assay was initiated by adding 5-6 x 10⁵ conidia (fungal suspension in DMEM medium) to 5-6 x 10⁴ macrophage cells in each well (MOI=10). The samples were then incubated in 5 percent CO₂ at 37°C.

The phagocytosis study procedure was the

following: after incubation for different time points (0, 30, 60, 120, and 240 minutes), the macrophage cells were washed with warm DMEM medium to remove an excess of conidia and then fixed by adding 2 percent (wt/wt) paraformaldehyde (PFD; Sigma-Aldrich GmbH, Steinheim, Germany) solution in PBS to the final concentration of 0.6 percent. The adherent macrophage cells were scraped and ten microliters of each sample was withdrawn to assess the percentage of phagocytosis (PP) which is the total number of macrophage cells from 100 cell count that can internalize fungal conidia. The phagocytic index (PI) was determined by counting the intracellular conidia and calculating for the average number of conidia per macrophage cell as follow:

$$\text{Phagocytic index (PI)} = \frac{\text{Total No. of intracellular conidia}}{\text{No. of phagocyte cells}}$$

To quantify killing activity, 0.5 percent sodium lauryl sulfate (SDS) (Sigma USA) was added to each well of the test and control for 3 minutes at room temperature. The SDS solubilized macrophage cells and the remaining particles were counted as by viable and nonviable conidia. Ten microliters of detergent-treated samples were diluted 200-fold in PBS, and 20 μ l was then plated in duplicate for colony forming unit (CFU) determination on SDA plates after 3-5 days incubation at 25-27°C. The percentage of killing in CFU was calculated as follow:

$$\text{Percentage of killing (PK)} = 100 \times \left(\frac{1 - \text{CFU test}}{\text{CFU control}} \right)$$

Statistical analysis

All data were expressed as mean \pm SD of the number of determinations carried out in triplicate for the percentage of phagocytosis and phagocytic index, and in duplicate for the percentage of killed CFU.

Variables were tested for normality and then the different groups were compared using the Paired Sample t-test, where $P < 0.05$ was considered as statistically significant between the groups.

RESULTS

Macroscopic and microscopic morphology

P. citrinum and *P. marneffei* were cultured on SDA agar at 28°C for 3 and 5 days, respectively. The yeast phase transformation of *P. marneffei* was performed by subculturing on brain heart infusion agar at 37°C for 7 days. After incubation, the fungi were observed under light microscope. Figure 1 shows their macroscopic and microscopic morphologies.

Phagocytosis assay

The results indicated a high efficiency of phagocytic activity of J774.1 macrophage cells against the conidia of *P. marneffei* and *P. citrinum*. Phagocytosis occurred when the conidia were incubated with the macrophage cells for 30 minutes at 37°C, and reached to the maximum after 120 minutes of incubation. In this study, the number of conidia phagocytosed by one macrophage was about 0-10 (Figure 2). The percentage of phagocytosis (PP) of *P. citrinum* was 47.00 \pm 2.65 percent at 30 minutes, 73.00 \pm 6.08 percent at 60 minutes, 80.00 \pm 3.00 percent at 120 minutes, and reached to the maximum of 88.00 \pm 2.65 percent at 240 minutes of incubation while the phagocytic index was 1.64 \pm 0.07, 2.09 \pm 0.07, 2.31 \pm 0.30, and 2.66 \pm 0.30, respectively. The PP of *P. marneffei* was 49.67 \pm 4.73 percent at 30 min, 72.33 \pm 3.51 percent at 60 minute, 90.67 \pm 9.29 percent at 120 minutes, and reached to the maximum of 93.67 \pm 7.51 percent at 240 minutes of incubation, while the PI was 1.60 \pm 0.21, 2.50 \pm 0.05, 3.98 \pm 0.68, and 4.57 \pm 0.44, respectively. There was no difference between the percentage of phagocytosis by *P. citrinum* and *P.*

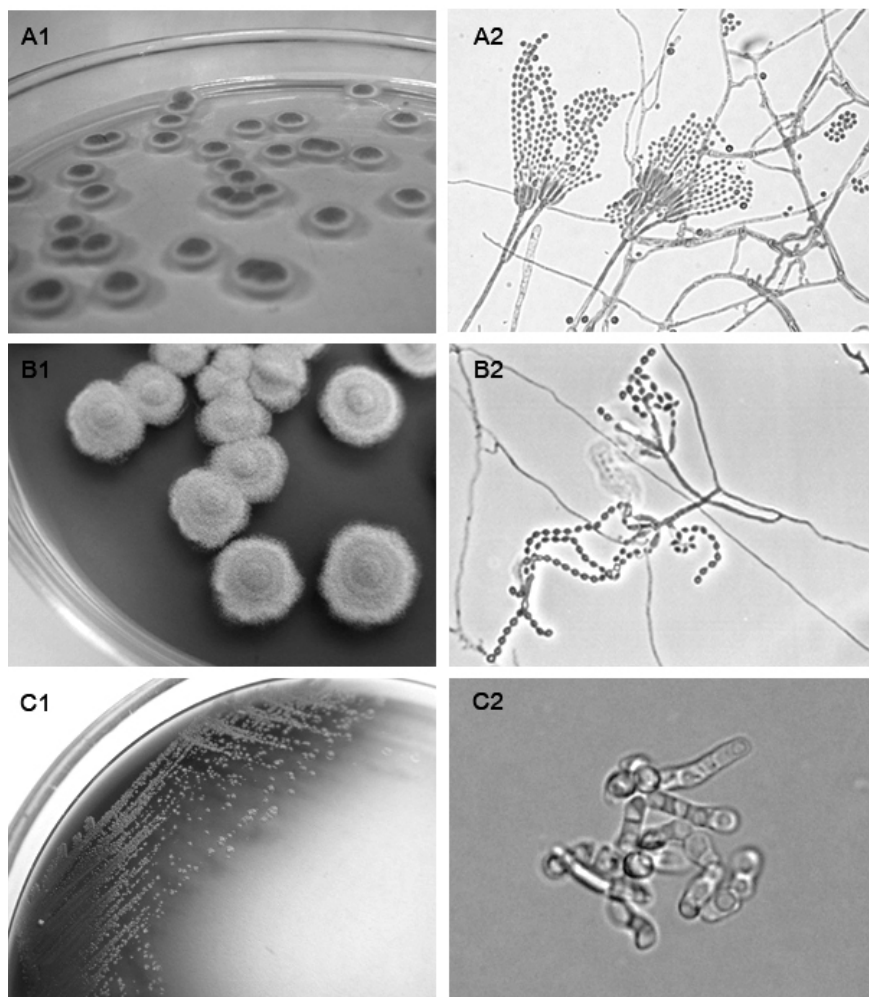


Figure 1. The macroscopic and microscopic morphologies of *Penicillium citrinum* cultured on Sabouraud's dextrose agar (SDA) for 3 days (A1-2), *Penicillium marneffeii* mold form cultured on SDA for 5 days (B1-2) and yeast form cultured on brain heart infusion (BHI) agar at 37°C for 7 days (C1-2) (magnification, 400).

marneffeii ($P > 0.05$) (Figure 3). However, the phagocytic indices of *P. marneffeii* at 60, 120 and 240 minutes of infection were higher than those of *P. citrinum* ($P < 0.05$) (Figure 4).

Killing assay

The killing activity of macrophages against *P. marneffeii* increased to approximately 7.23 ± 1.22 percent, 16.55 ± 6.05 percent, 38.85 ± 6.94 percent, and 61.71 ± 1.54 percent after 30, 60, 120 and 240 minutes of incubation, respectively. In the non-

pathogen, *P. citrinum*, 42.40 ± 4.23 percent, 68.56 ± 3.03 percent, 66.78 ± 2.48 percent and 64.12 ± 3.42 percent of conidia were killed after 30, 60, 120, and 240 minutes of incubation, respectively. In the early stage of phagocytosis, the percentage of killing of *P. citrinum* was significantly higher than those observed in pathogenic *P. marneffeii* ($P < 0.05$) (Figure 5).

DISCUSSION

P. marneffeii, a pathogenic dimorphic fungus, can cause disseminated disease in people with impaired cell-

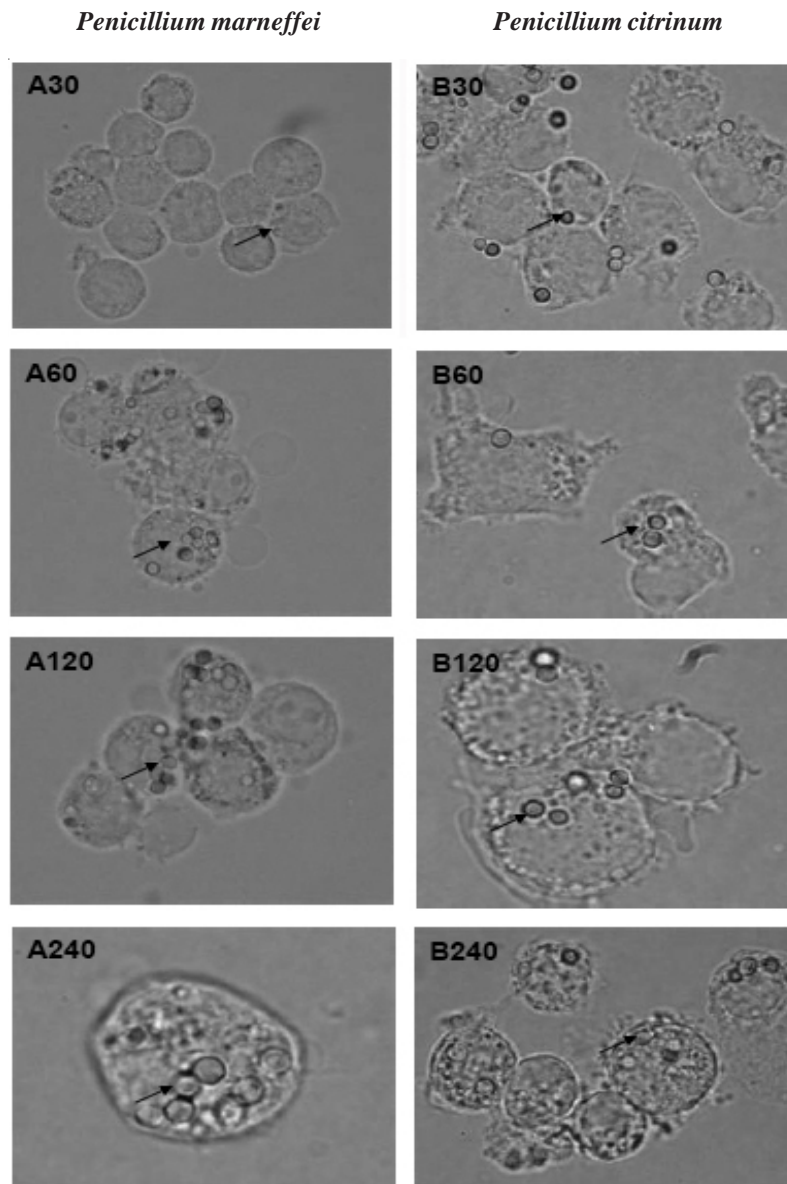


Figure 2. Phagocytosis of *Penicillium marneffe* and *Penicillium citrinum* conidia by mouse macrophage J774.1 cells. Microscopic observation was performed at time 30, 60, 120, and 240 minutes (columns A and B, respectively). There is no phagocytosis at the time zero (data not shown). At 30 minutes of incubation, all conidia attached the macrophage cells (A30) and some intracellular conidia are seen (B30). More internalized conidia in the macrophages are seen after longer incubation time (A60-240 and B60-240). At 240 minutes after incubation, some of *P. citrinum* conidia are lysed inside the macrophages (B240). The intracellular conidia were counted and calculated for percentage of phagocytosis (PP) and phagocytic index (PI). The arrows indicate the intracellular conidia (magnification, 1,000).

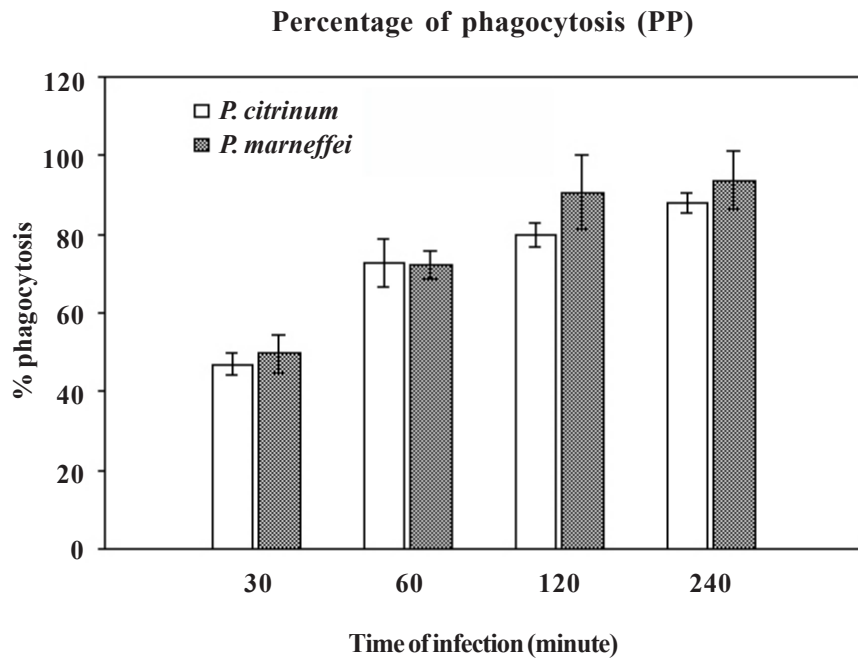


Figure 3. The percentage of phagocytosis (PP) of *Penicillium citrinum* (white) and *Penicillium marneffei* (black). The phagocytosis occurred at 30 minutes of incubation and reached maximum at 240 minutes. There was no difference in the PP values of both fungi ($P>0.05$).

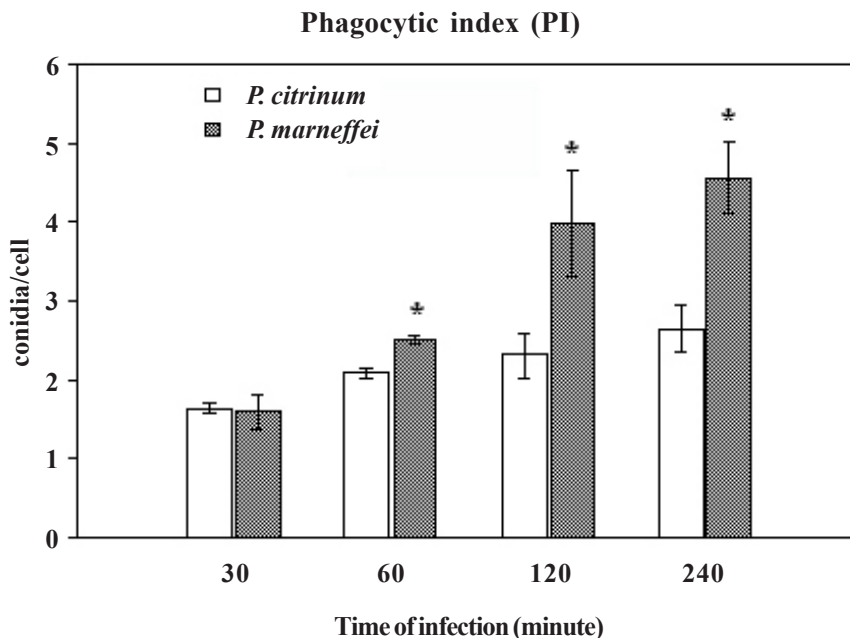


Figure 4. The phagocytic index (PI) of *Penicillium citrinum* and *Penicillium marneffei*. The phagocytosis occurred at 30 minutes of incubation, and the PI results were time dependent. The graph indicated that the PI of *P. marneffei* (black) was significantly higher than that of *P. citrinum* (white) at 60, 120, and 240 minutes of infection ($*P<0.05$).

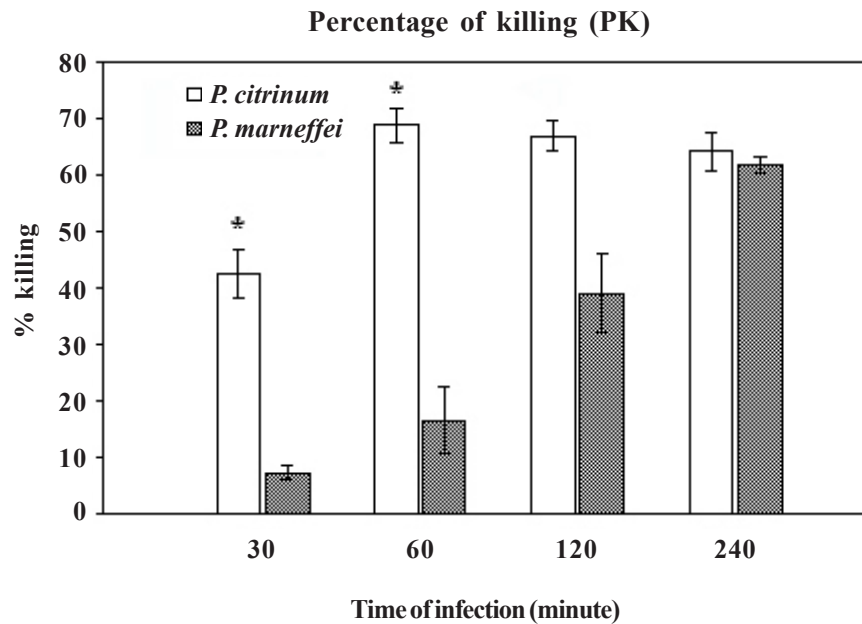


Figure 5. The percentage of killing of *Penicillium citrinum* and *Penicillium marneffeii*. The killing activity of the macrophage cells against the conidia of *P. citrinum* (white) was significantly higher than that of *P. marneffeii* (black) at the early incubation time of 30 and 60 minutes (* $P < 0.05$).

mediated immunity, especially those infected with HIV. The types of exposure and route of entry of this fungus, leading to human infection, are still unclear. By analogy with other opportunistic fungal pathogens, it seems quite likely that conidia may be inhaled from a contaminated environment and subsequently disseminate from the lungs when the host is immunosuppressed.⁶⁻⁷ During infection, phagocytic cells are likely to be the primary line of the host defense against this fungus. In phagocytes, the fungus can survive and proliferate in the phagosome. They also demonstrated that histiocytes of tissues infected with *P. marneffeii* contained yeast-like cells of *P. marneffeii*. As the lesions progressed, the intracellular fungal cells were released following cellular necrosis and subsequent abscess formation. Free fungal cells or phagocytes containing fungal cells can then disseminate throughout the body.

In this study, the J774.1 murine macrophage-like cell line, which was found to be appropriate for *in vitro*

studies of *P. marneffeii*, was used.⁴ *In vitro* phagocytosis and the killing activity of mouse macrophage J774.1 cells against the conidia of pathogenic *P. marneffeii* were examined, compared with non-pathogenic *P. citrinum*. The collective results of this study suggest that macrophages play a crucial role in the immune response to the fungi. Differences in the percentage of phagocytosis of both pathogenic and non-pathogenic *Penicillium* isolates used in this study by J774.1 cells were not statistically significant. However, the phagocytic indices of *P. marneffeii* were higher than those of *P. citrinum* after incubation at 60 minutes because a number of *P. citrinum* conidia were lysed inside the macrophages (Figure 2, B120-240). Coincidentally, the percentage of killing results of *P. citrinum* was reached maximum at 60 minutes of incubation (Figure 5). This result revealed that *P. marneffeii* was more susceptible to being phagocytosed by macrophage J774.1 cells than *P. citrinum*. Although,

the slightly increase of percentage of phagocytosis and phagocytic index of both fungi could be observed, the phagocytic activity of macrophages would be steady after 240 minutes prolonged incubation. But the conidia of *P. marneffeii* were also more resistant to being killed by macrophages than the non-pathogenic fungus, *P. citrinum*. These results suggest that *P. marneffeii* conidia are able to inactivate macrophage defenses. Phagocytosis of microbes by macrophages normally results in microbe elimination through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the action of lysosomal enzymes following phagolysosomal fusion.⁸⁻⁹ Thus, the survival within the phagocyte environment of *P. marneffeii* may depend on some virulence mechanisms that counter or inactivate macrophage defenses, and diverse mechanisms likely exist for the dimorphic transition.⁴ In contrast, the conidia of non-pathogenic *P. citrinum* could not escape from these defense mechanisms and were readily killed by macrophage cells. In many respects, macrophage interactions with *P. marneffeii* are quite similar to that seen with *Histoplasma capsulatum*. Yeast cells from both species are bound and phagocytosed in the absence of opsonin and both stimulate a respiratory burst.¹⁰⁻¹² Similarly, the pathophysiology and clinical presentations of histoplasmosis and penicilliosis marneffeii share overlapping features including the parasitism of macrophages.^{6,13-14} However, clearly there are differences, most notably in the mechanisms by which the two fungi gain access to the macrophage. *H. capsulatum* entry into macrophages is divalent cation dependent and uses CD18 complex^{10,12}, whereas *P. marneffeii* is divalent cation independent and gains access through a lectin wheat germ agglutinin (WGA) inhibitable process.¹⁵ However, many unanswered questions remain, including the definition of the specific receptor(s) responsible for binding and the conditions

necessary to activate the macrophage to inhibit and kill *P. marneffeii*. Moreover, the study of interactions between macrophages and *P. marneffeii* could serve to increase general understanding of the mechanisms of intracellular parasitism. The mechanism of intracellular infection and survival inside the macrophages of *P. marneffeii* should be investigated further.

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