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Distinct Immunologic Properties of *Penicillium marneffei* Yeasts Obtained from Different *in vitro* Growth Conditions

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SUMMARY A dimorphic fungus *Penicillium marneffei* is a causative agent of penicilliosis, a life-threatening disseminated disease in immunocompromised hosts predominantly found in southeast Asia and southern China. *P. marneffei* is the only known *Penicillium* that possesses a dimorphic characteristic. Since it is difficult to produce large amount of *P. marneffei* yeasts *in vivo* for experimentation purpose, yeast cells were produced in different *in vitro* conditions as alternatives. We interested in investigating the immunologic properties of yeast cells from different culture preparations. It was found that yeast cells obtained from brain heart infusion broth and Sabouraud dextrose broth did not resemble those resided in clinical specimens. A solution of 1% peptone, on the other hand, could induce a direct conidial transition into fission yeasts. Ability of yeast cells in each preparation to activate macrophages was determined by analyzing surface expression of CD40 and CD86 co-stimulatory molecules after two days of co-cultivation. Every *P. marneffei* yeast cell preparation demonstrated such ability. However, the ones from Sabouraud dextrose broth seemed to induce less phagocytosis. Additionally, although distinct antigenic profiles and lack of conformity in antigenic expression were observed among yeast cells from different culture conditions, most major immunogenic bands were present when Western analysis was performed using polyclonal antisera from penicilliosis patients. The results of the study raise attention on immunological and biochemical characteristics of *P. marneffei* yeasts if such preparations are to be used in future laboratory investigations.

Penicillium marneffei is an opportunistic pathogenic fungus in southeast Asia and southern China.[1-4] It inhabits reticuloendothelial system of humans and bamboo rats.[3,5] With the spread of human immunodeficiency virus into northern Thailand, approximately 1,115 cases of penicilliosis were reported at Chiang Mai University Hospital, Chiang Mai, Thailand, over a period of 7 years.[3] The most common clinical features of the disease included fever, anemia, weight loss, generalized lymphadenopathy, hepatosplenomegaly and cough.[1-4] Organs involved in the infection included lungs, liver, spleen and skin. In biopsy specimens, diffused infiltration of macrophages engorged with proliferating yeast cells in tissue was frequently observed.[1-4]

P. marneffei displays a temperature dependent dimorphism that is the ability to produce either multi-nucleated septate hyphae at 25°C or uninucleated yeast cells at 37°C.[6,7] The yeast cells are generated by a process called arthroconidiation where discernable double septa are formed between short hyphal cells of approximately 20 μm in length (pre-arthroconidial cells).[8] Subsequently, the cell wall materials between the double septa are degraded resulting in liberation of uninucleated single cells designated arthroconidia. Arthroconidia initiate polarized growth and divide by fission after nuclear division to produce the true yeast cells.[8]

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As *P. marneffei* dimorphic switch is triggered by temperature, the method for preparing an inoculum of yeast cells in brain heart infusion (BHI) broth at 37°C for 7-14 days, described by Sekhon *et al.*,[9] was generally used in previous *P. marneffei* studies.[8] However, from our experience, not all isolates could generate arthroconidia or uninucleated yeasts when this cultivation method was introduced.[10] In addition, a homogeneous culture of yeast cells of *P. marneffei* isolates that were transitionally capable has never been observed in this medium. Therefore, despite the transition under temperature change, other factors that can trigger the switch of *P. marneffei* to yeast phase should be investigated. In this study, we have examined growth and transition of *P. marneffei* conidia to yeasts in various conditions and differences in immunogencity and antigenic profiles among preparations were observed. In addition, *P. marneffei* yeasts obtained from different media demonstrated distinct abilities in the induction of phagocytosis. These observations raise awareness for future investigation on immune response to *P. marneffei* if such yeast preparations are to be used.

MATERIALS AND METHODS

Microorganisms and sources

A clinical isolate and a standard strain of *P. marneffei* were used in this study. Isolate RT72 obtained from an AIDS patient, whose hemoculture was positive for penicilliosis, was described elsewhere.[10] The standard strain ATCC 64102 was purchased from American Type Culture Collection, Rockville, MD. Every isolate was cultured on malt extract agar (Difco) or potato dextrose agar (Difco) at 25°C for 2-3 weeks. To collect conidia, 2 ml of 0.01% Tween-80 (Sigma-Aldrich, St. Louis, MO) in sterile distilled water were added to each culture at late exponential phase. After harvesting, the concentration of conidia was estimated by means of hemacytometer counts.

Media

Basal media used in the study were brain heart infusion broth (BHI, Difco), Sabouraud dextrose broth (SDB, Difco) and yeast nitrogen base (YNB, Becton Dickinson, Sparks, MD). The media were prepared according to manufacturer's recommendation. BHI was used as the standard medium for comparison of phase switching into yeast form.[9] To observe the effects of yeast extract (YE) as well as peptone (Difco) on growth and phase transition, different concentrations of YE and peptone were added separately as supplements to the media (1%, 2%, 4% final concentrations).

Induction of yeast-phase switching and growth measurement

Approximately 10^8 *P. marneffei* conidia/ml were added to 50 ml of each tested medium and the cultures were incubated in a 37°C gyratory shaker at 120 rpm. The culture was observed for the total time of 9-12 days. Number of yeast cells was counted microscopically under 20x objective using hemacytometer. Each isolate was tested on 5 independent occasions in duplicate.

Phagocytosis of yeast cells

Blood were collected from healthy donor and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll-Hypaque density gradient (Nycomed Pharma, Oslo, Norway). PMBCs were washed three times with PBS and resuspended in 10% FBS-supplemented RPMI 1640 (complete medium, Gibco BRL). Then PBMCs at 2.5 x 10^6 cell/ml were plated onto a 2-ml Petri dish and the dish was kept in a CO_2-incubator for 24 hours at 37°C. After discarding non-adhered cells, seven-day old *P. marneffei* yeasts were added to the adhered monocyte-derived macrophages. The macrophages and yeast were co-cultured at the multiplicity of infection of 5 in the complete medium at 37°C for 2 days. Then, the cells were fixed with 2% glutaraldehyde and stained with Giemsa. Phagocytized yeasts were observed and photographed under Olympus light microscope.

Flow cytometry

In a parallel experiment to the phagocytosis of *P. marneffei* yeasts, monocyte-derived macrophages with phagocytized yeast cells were scraped with a cell scraper (TechnoPlastic Products AG, Transadingen, Switzerland). The cells were harvested by centrifugation at 1,000 x *g* and resuspended in 50 µl RPMI-1640. Five microliters of phycoerythrin (PE)-labeled monoclonal antibody to CD86 and allophycocyanin (APC)-labeled monoclonal antibody to CD40 (Becton Dickinson Biosciences Pharmingen, San Diego, CA, USA) were added to the harvested cells and the suspension was left at 4°C for 30 minutes. Subsequently, the cells were washed with PBS once and fixed with 2% formaldehyde for 30 minutes. The CD40 and CD86 expres-

sions were determined as mean fluorescent intensity (MFI) by FACSCalibur (Becton Dickinson, Mountain View, CA, USA), and phagocytosis activity was determined by a plot of side scatter.

Comparison of protein profiles of different yeast cell preparations

P. marneffei conidia were cultured in 1% peptone, BHI or SDA supplemented with 4% yeast extract at 37°C in a gyratory shaker. *P. marneffei* yeasts grown in BHI and SDB + 4% YE were harvested on Day 6 while those grown in peptone were harvested on Day 7. The yeast cells were washed three times and subsequently frozen at -20°C. Supernatant was collected, dialyzed in two changes of PBS and subsequently once in 0.1 N $(NH_4)HCO_3$ using a dialysis bag with the molecular weight cutoff at 10,000 Daltons. The dialyzed supernatant was lyophilized using Heto FD 8.0 Lyophilizer (Heto-Holten, Allerød, Denmark) and kept at -20°C until used.

Yeast cells of 10^8 cells and lyophilized supernatant of 200 μg from different preparations were suspended in reducing sample buffer containing 5% (v/v) 2-mercaptoethanol. The samples were heated at 100°C for 5 minutes and subsequently subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[11] Western blot analysis was performed employing anti-*P. marneffei* polyclonal antibodies from AIDS patients infected with *P. marneffei* strains RT-18 and AR-22 as well as a monoclonal antibody 3C2 which recognized a 38 kDa protein described previously.[12]

RESULTS

Transition of *P. marneffei* conidia into yeasts in different media

Previously, BHI at 37°C has been used to initiate morphological transition of *P. marneffei* hyphae to yeasts.[8, 9] *In vitro* yeast transition in this particular medium always results in mixed population of yeast cells and short hyphae (Fig. 1A). Similar result was also observed when conidia were cultured in SDB in that the cells were heterogeneous in size and shape. Conversion of *P. marneffei* conidia to yeast cells in both media began with apical growth of conidia to form hyphae and followed by cell division to generate arthroconidia. Maximal growth in both BHI and SDA media was obtained at days 5-6.

With 4% YE supplemented in SDB, the medium gave the highest numbers of yeast cells, although similar in size and shape, when compared to SDB alone and SDB supplemented with 1% and 2% YE (data not shown). Moreover, the yeast cells converted under the SDB with 4% YE medium appeared healthier and more homogeneous (Fig. 1B). Generally, yeast cells generated in SDB with 4% YE were larger in size when compared to those generated in the BHI medium (Fig. 1A and 1B).

Characteristics of yeast cells in YNB media with 1% peptone and in 1% peptone dissolved in distilled water (Fig. 1C) were compa-

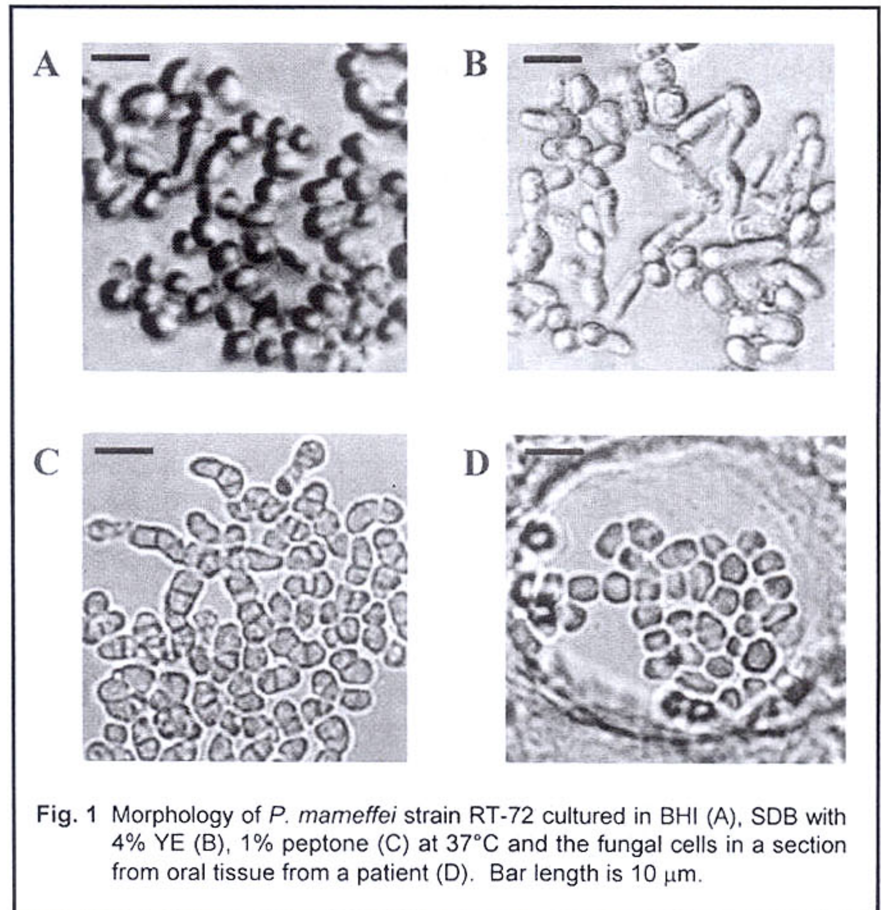

Fig. 1 Morphology of *P. mameffei* strain RT-72 cultured in BHI (A), SDB with 4% YE (B), 1% peptone (C) at 37°C and the fungal cells in a section from oral tissue from a patient (D). Bar length is 10 μm.

rable to those found in oral biopsy tissue of an AIDS patient (Fig. 1D). The germinated conidia did not elongate to form hyphae and arthroconidiation was not observed. On the other hand, they seemed to grow and then divided by septation to form fission yeast.

Immunogenicity of yeast cells from the three preparations

To examine the immunogenicity of yeasts generated from the three distinct media, host immune cells such as macrophages derived from PBMCs were activated with the *P. marneffei* yeast cells. PBMCs-derived macrophages were stimulated with different yeast cell preparations for 8 hours, 1 day and 2 days. Adhesion of yeast cells to macrophages was observed 2 hours after initial incubation and yeast cells were detected intracellularly after 18-24 hour co-cultivation (Fig. 2). Cellular activation was determined by monitoring the expression of co-stimulatory molecules, CD40 and CD86. After 8 hour and 1 day incubation, no difference in the expression of co-stimulatory molecules was observed among the stimulated macrophages in comparison to the non-stimulated controls. However, the expression of CD40 and CD86 increased after 2 days of co-culturing. The mean fluorescent intensities (MFIs) of CD 86 were 12.45 and 94.57 for IgG isotype controls and non-activated macrophages, respectively. The MFIs of CD86 expression levels on activated macrophages were 364.19 for those incubated with yeasts from SDB with 4% YE, 453.75 for those co-cultured with yeasts from BHI and 449.77 for those activated with yeasts from 1% peptone (Fig. 3A). Similar results were observed on the MFIs of

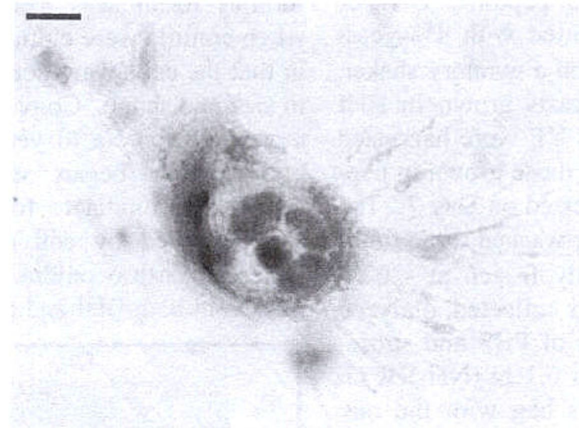

Fig. 2 Giemsa staining of a macrophage that phagocytized *P. marneffei* yeasts. Bar length is 5 μm.

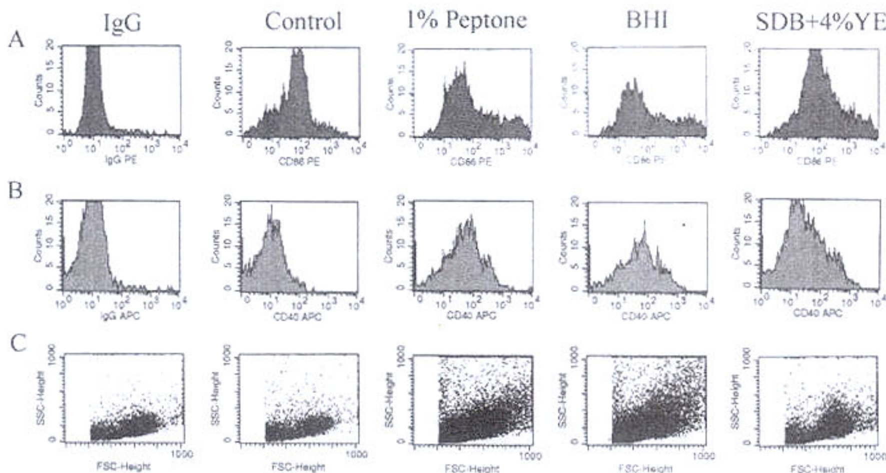

Fig. 3 Flow cytometric analysis. Expression of CD86 (A) and CD40 (B) co-stimulatory molecules as well as FSC/SSC analysis (C) of macrophages which were co-cultured with *P. marneffei* yeasts from 1% peptone, BHI and SDB + 4% YE are depicted. "Control" indicates non-activated macrophages whereas "IgG" indicates non-activated macrophages stained with an isotype-specific monoclonal antibody in immunofluorescence experiment.

CD40 in that they were at 15.23 and 12.32 for IgG isotype controls and non-activated macrophages, respectively. The MFIs of CD40 expression on activated populations were 74.91 for those incubated with yeasts from SDB with 4% YE, 108.06 for those co-cultured with yeasts from BHI, and 96.98 for those activated with yeasts from 1% peptone (Fig. 3B). Taken together, it was demonstrated that *P. marneffei* yeasts induced by different conditions could equally activate the PBMCs-derived macrophages judging from an increase in the expression of the co-stimulatory molecules.

Additionally, phagocytic activity of the PBMCs-derived macrophages was determined by a plot of size scatter. Cells with high granularity represent actively phagocytized macrophages. Macrophages were shown to actively ingest yeast cells from BHI and 1% peptone cultures (Fig. 3C). In contrast, the yeast cells cultured in SDB with 4% yeast extract seemed to induce less phagocytosis as determined by the lower number of macrophages with high granularity (Fig. 3C).

Antigenic profiles of different yeast cell preparations

Harvested yeast cells from 1% peptone (Fig. 4, Lane A), BHI (Fig. 4, Lane B) and SDB with 4% YE (Fig. 4, Lane C) as well as three batches of culture supernatant from the cultures (Fig. 4, Lane D from 1% peptone; Lane E from BHI; Lane F from SDB with 4% YE), at equal concentrations, were analyzed through SDS-polyacrylamide gel electrophoresis and the protein bands were electrophoretically transferred to nitrocellulose membrane. The membranes were then incubated with sera from penicilliosis patients RT-18 (Fig. 4, left panel) and AR-22 (Fig. 4, middle panel). Another set of membrane blotted antigens was exposed to MAb 3C2 (Fig. 4, right panel). With the same number of yeast cells, higher concentrations of glycoprotein antigens were detected from yeasts cultured in SDB supplemented with 4% YE whereas similar levels of the production were observed in cultures of BHI and 1% peptone. Thickness of the 38 kDa band from the Western blot analysis using MAb 3C2 (Fig. 4, right panel) confirmed the fore mentioned findings. Overall, most major antigenic bands were present in all three antigenic preparations. However, the levels of expression of certain proteins such as those of molecular weights ~79 kDa (Fig. 4, left panel), ~140 kDa and ~53 kDa (Fig. 4, middle panel) were distinct among different preparations. Lack of conformity in the levels of antigenic expression of yeast cells from different preparations was also found when other patients' sera were used for the detection (data not shown).

DISCUSSION

P. marneffei is a dimorphic fungus. Its life cycle can be divided into two stages: filamentous vegetative growth with asexual reproduction (conidiation) at 25°C and unicellular yeast vegetative growth at 37°C. Its yeast form is the pathogenic form that is found in clinical specimen.[6,13] An *in vitro* study by Cogliati *et al.*[14] in 1997 also showed

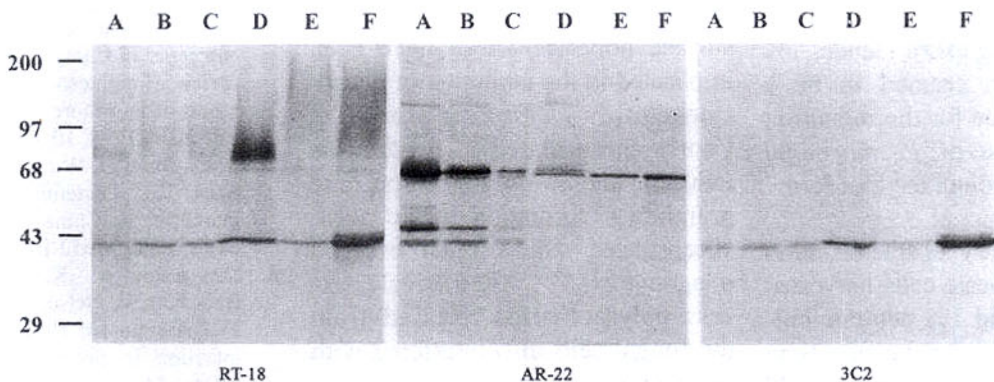

Fig. 4 Western blot analyses of whole cell preparations and lyophilized culture supernatant samples against polyclonal (RT-18, AR 22) and monoclonal (3C2) antibodies. Lanes A, B and C are whole cell preprations of yeasts from 1% peptone, BHI and SDB+4% YE, respectively. Lanes D, E, and F represent batches of culture supernatant obtained from the yeast cultures in 1% peptone, BHI and SDB+4% YE, respectively.

that *P. marneffei* conidia, once phagocytized by the J774 murine macrophage-like cell line, replicated by fission within 24 hours to form yeast cells. Therefore, it is of relevance to develop a methodology to induce morphogenic switching of *P. marneffei* conidia to fission yeasts *in vitro* for the study of pathogenesis of *P. marneffei* infection.

Previously, BHI is a medium used to generate *P. marneffei* yeast cells in most studies.[15,16] Yeast cells harvested from the BHI culture developed through arthroconidiation. The formation of yeast cells in the BHI medium occurs during the late stage of growth. Thus, the BHI culture always contains yeast cells as well as arthroconidia mixed with dead cells. In the present study, it has been shown that conidia grown in SDB with 4% YE displayed similar growth characteristics as those grown in BHI at 37°C while 1% peptone broth at 37°C converted conidia into yeast cells within 24 hours and a homogeneous population of fission yeasts was obtained thereafter. Microscopically, the size and shape of the yeast cells obtained from this medium were also similar to those found in the clinical specimen (Fig. 1D). Hence, 1% peptone solution seemed to be a medium-of-choice for the induction and maintenance of *P. marneffei* growth from conidium to yeast form.

Preliminary characterization of *P. marneffei* yeast cells harvested from the BHI and 1% peptone culture demonstrated for the first time that the yeast cell populations could stimulate PBMCs-derived macrophages to express co-stimulatory molecules and could enhance phagocytic activity of the macrophages. Based on the two signal model, co-stimulatory molecule expression on the surface of antigen presenting cells such as macrophage is critical for T cell activation. In the absence of co-stimulation, T cells triggered by T cell receptor become anergic.[17] In the case of *Cryptococcus neoformans*, Vecchiarelli *et al.*[18] also demonstrated that its yeast form could induce CD80 and CD86 expression on human monocytes but the magnitude of expression is dependent on yeast encapsulation.[18] Encapsulated yeast cells stimulated less co-stimulatory molecule expression than acapsular ones.

In comparison, yeast cells from the SDB culture induced comparable levels of co-stimulatory molecule expression but less phagocytosis by PBMCs-derived macrophages. Given that SDB consists of higher percentage (4% w/v) of dextrose than BHI (2% w/v) and 1% peptone (none),[19] transition of *P. marneffei* conidia in these different nutritional environments would result in the generation of yeast cell populations of distinct biochemical natures. Such alteration might affect opsonic property of certain surface molecules thereby interfering with the phagocytic induction. Previously, surface polysaccharides have been implicated in the inhibition of phagocytosis of *C. neoformans* yeasts.[20,21] Poorly encapsulated *C. neoformans* induced intense inflammation and granuloma formation while well-encapsulated strains often escaped phagocytosis.[20,21] Alternatively, excess polysaccharide released from the fungal cells also interfered with phagocytosis.

The results of this study demonstrated that *P. marneffei* yeasts generated from conidia in different *in vitro* conditions displayed distinct morphologies and immunologic characteristics. Nevertheless, *P. marneffei* yeasts obtained from BHI and 1% peptone cultures were quite immunogenically conformed. Since recovery of yeast cells from infected tissues and animals were relatively difficult, further characterization on biochemical, immunological, and molecular nature is necessary to confirm that the yeast cells generated *in vitro* could represent those reside in infected macrophages and tissues. Finally, transition of conidia into yeasts in 1% peptone is an alternative cultivation method in that a homogeneous culture of fission yeasts with similar antigenic profiles as those generated from BHI is obtained.

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