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Melanization of *Penicillium marneffei in vitro* and *in vivo*

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Melanins are found universally in nature and are implicated in the pathogenesis of several important human fungal pathogens. This study investigated whether the conidia and the yeast cells of the thermally dimorphic fungal pathogen *Penicillium marneffei* produce melanin or melanin-like compounds *in vitro* and during infection. Treatment of conidia with proteolytic enzymes, denaturant and concentrated hot acid yielded dark particles that were similar in size and shape to the conidia. A melanin-binding monoclonal antibody (mAb) labelled pigmented conidia, yeast cells and the isolated particles as determined by immunofluorescence microscopy. Electron spin resonance spectroscopy revealed that particles derived from pigmented conidia contained a stable free radical compound, consistent with their identification as melanins. Skin tissue from penicilliosis marneffei patients contained yeast cells that were labelled by melanin-binding mAb. Additionally, sera from *P. marneffei*-infected mice developed a significant antibody response (both IgG and IgM) against melanin. Phenoloxidase activity capable of synthesizing melanin from L-DOPA was detected in cytoplasmic yeast cell extracts. These findings indicate that *P. marneffei* conidia and yeast cells can produce melanin or melanin-like compounds *in vitro* and that the yeast cells can synthesize pigment *in vivo*. Accordingly this pigment may play some role in the virulence of *P. marneffei*.

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INTRODUCTION

Penicillium marneffei is a dimorphic pathogenic fungus which causes human infection in Southeast Asia, particularly in Thailand (Chiewchanvit *et al.*, 1991; Supparatpinyo *et al.*, 1994), Hong Kong (Tsang *et al.*, 1991) and southern China (Li *et al.*, 1991). Although this fungus can cause infection in both immunocompetent and immunocompromised hosts, penicilliosis caused by *P. marneffei* in the non-HIV-infected population is extremely rare (Jayanetra *et al.*, 1984; Deng *et al.*, 1988). Since the arrival of the AIDS epidemic, the number of cases occurring in immunocompromised individuals has risen dramatically in Southeast Asia (Chiewchanvit *et al.*, 1991; Supparatpinyo *et al.*, 1994). In northern Thailand, disseminated penicilliosis is ranked as the third most common AIDS-defining illness in Thailand, after tuberculosis and cryptococcosis (Supparatpinyo *et al.*, 1994). In AIDS patients, infection with *P. marneffei* presents as a disseminated illness with fever, weight loss, skin lesions and pancytopenia (Tsui *et al.*, 1992; Sirisanthana & Sirisanthana, 1995), and it is fatal if untreated.

Abbreviations: DHN, dihydroxynaphthalene; DOPA, 3,4-dihydroxyphenylalanine; ESR, electron spin resonance spectroscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

P. marneffei grows as a saprophytic filamentous mould containing numerous conidia at room temperature and as a yeast-like fungus in living tissue or in culture at 37 °C. The organism has been isolated from bamboo rats and from soil around the animals' nesting areas (Chariyalertsak *et al.*, 1996), although its precise ecological niche remains uncertain. The likely route of infection in most cases is inhalation of *P. marneffei* conidia. Presumably, the ability of conidia to persist within the host is fundamental to the establishment of infection.

Melanins are multifunctional compounds found in diverse species that include representatives of all biological kingdoms (Hill, 1992). Melanins have been implicated in virulence in human fungal pathogens; thus DHN-melanin has been shown to be an important virulence determinant in *Wangiella dermatitidis* (Dixon *et al.*, 1987; Jacobson & Tinnell, 1993; Jacobson *et al.*, 1995; Schnitzler *et al.*, 1999), *Sporothrix schenckii* (Romero-Martinez *et al.*, 2000; Morris-Jones *et al.*, 2003) and *Aspergillus fumigatus* (Jahn *et al.*, 1997, 2000; Langfelder *et al.*, 1998; Tsai *et al.*, 1998, 1999). One factor associated with the protection of conidia and hyphae of *W. dermatitidis* from the host immune response is the presence of fungal melanin (Wheeler & Bell, 1988). The protective mechanism exerted by the melanin remains

unclear, although it has been shown that fungal melanin protected this pathogen from being killed in the phagolysosome of neutrophils. However, melanin did not inhibit phagocytosis or the oxidative burst of the neutrophils involved (Schnitzler *et al.*, 1999). Furthermore, melanin-deficient mutants of *W. dermatitidis* exhibited lower degrees of virulence and slower invasive hyphal growth than melanized wild-type strains (Brush & Money, 1999). *In vitro* and *in vivo* studies (Feng *et al.*, 2001) have determined that melanin-deficient mutants of *W. dermatitidis* are more susceptible to the killing mechanisms of human granulocytes and they exhibit severely reduced virulence in murine models of infection. In *A. fumigatus*, pigmentless mutant conidia have been shown to be more susceptible to hydrogen peroxide and sodium hypochlorite (Jahn *et al.*, 2000), and are more efficiently damaged than pigmented conidia by human monocytes *in vitro* (Jahn *et al.*, 1997). In addition, melanin in *A. fumigatus* conidia is able to quench reactive oxygen species, a major host antimicrobial effector system derived from human granulocytes (Jahn *et al.*, 2000).

In contrast, *Cryptococcus neoformans* produces DOPA-melanin; in this pathogen, melanized yeast cells are less susceptible than nonmelanized cells to UV-induced damage (Wang & Casadevall, 1994a), macrophage-mediated damage (Wang & Casadevall, 1994b; Blasi *et al.*, 1995), oxidant-mediated damage (Wang *et al.*, 1995), antimicrobial peptides (Doering *et al.*, 1999), heavy metal toxicity (Garcia-Rivera & Casadevall, 2001) and anti-fungal drugs such as amphotericin B (Wang & Casadevall, 1994c).

No previous substantive efforts have been made to detect melanization in *P. marneffei*. Given the potential role of fungal melanin in virulence, we have investigated whether conidia and yeasts of *P. marneffei* synthesize melanin or melanin-like compounds by utilizing techniques developed to study and isolate melanin from other fungal pathogens.

METHODS

Production of *P. marneffei* conidia and yeast cells. *P. marneffei* ATCC 200051 was isolated from a bone marrow sample of a patient infected with HIV at Chiang Mai University, Chiang Mai, Thailand, and was maintained by monthly subculture onto slants of Malt Extract Agar (MEA; Oxoid). *P. marneffei* isolates NCPF 4060 and NCPF 4062 were obtained from blood cultures via the National Collection of Pathogenic Fungi, Colindale, London, UK, and were used only in the production of antigen for laccase activity. *P. marneffei* was grown on MEA at 25 °C for 10 days and the conidia were then collected by adding 5 ml sterile PBS to the culture tubes; conidia were removed by gentle scraping with a plastic loop. The conidia were collected by filtration through sterile glass wool, centrifuged at 8000 ***g*** for 30 min, and then washed three times with sterile PBS. For yeast cells, *P. marneffei* was cultured in Brain Heart Infusion Broth (BHI; Oxoid), at 37 °C for 7 days with continuous shaking at 120 r.p.m. To harvest the yeast, the broth cultures were centrifuged at 10 000 ***g*** for 30 min, and the pellet was washed three times with sterile PBS. In addition, yeast cells from NCPF 4060 and 4062 were produced for production of cytoplasmic antigens.

Preparation of slide cultures of *P. marneffei* ATCC 200051. Slide cultures were prepared in sterile Petri dishes by placing slides on sterile glass rods. Sabouraud dextrose agar (SDA) blocks cut into 1 cm cubes were put on the slides and a small amount of the fungus was inoculated to each of four sides of the agar block. A sterile cover slip was placed on the top of the inoculated agar block. For humidification, sterile cotton wool was put in the plate and 2–3 drops of water were added. Plates were then sealed with masking tape and kept at room temperature for 2 weeks. Slides were periodically removed from the Petri dish and examined. When conidiation was complete, the cover slip was removed and the agar block was lifted from the slide (McGinnis, 1980). Before processing further, absolute ethanol was added to the adherent fungi on the slide, and the slides were then air-dried.

Isolation and purification of melanin particles from *P. marneffei* conidia and yeast. *P. marneffei* ATCC 200051 was grown on MEA slants for 10 days at 25 °C and conidia were collected as described. For yeast cells, *P. marneffei* was cultured in BHI broth for 7 days at 37 °C and yeast cells were then harvested as described. Conidia and yeast cells were washed three times with PBS, with a final wash with 1·0 M sorbitol and 0·1 M sodium citrate (pH 5·5). Novozyme (cell-wall-lysing enzymes from *Trichoderma harzianum*; Sigma) was added at 10 mg $ml^{-1}$and incubated overnight at 30 °C to generate protoplasts. The protoplasts were collected by centrifugation, washed three times with PBS, and incubated in 4·0 M guanidine thiocyanate (Sigma) overnight at room temperature. The resultant dark particles were collected by centrifugation, washed three times with PBS, and treated with 1·0 mg Proteinase K ml^{-1} (Roche) in reaction buffer [10·0 mM Tris, 1·0 mM $CaCl_2$ and 0·5 % (w/v) SDS, pH 7·8] and incubated at 37 °C. The resultant debris was washed three times with PBS and boiled in 6·0 M HCl for 1·5 h. After treatment by boiling in acid, melanin particles were collected by filtration through Whatman paper no. 1 and were washed extensively with distilled water. Melanin particles were then dialysed against distilled water for 10 days until the acid was completely removed, whereupon they were lyophilized.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Briefly, for TEM, the melanin particles were fixed in 2 % (v/v) glutaraldehyde for 2 h, then incubated overnight in 4 % (v/v) formaldehyde/1 % (v/v) glutaraldehyde/0·1 PBS, and subjected to 1·5 h post-fixation in 2 % (v/v) osmium tetroxide. Dehydration was accomplished by serial incubation in graded ethanol and two final incubations in 100 % ethanol. The melanin particles were embedded in Spurr's resin. TEM pictures were then obtained with a Hitachi H 7600 transmission electron microscope. For SEM the melanin particles were fixed overnight in a 4 % (v/v) glutaraldehyde solution in PBS, transferred to polylysine-coated coverslipes, dehydrated by incubation in graded ethanol, mounted with gold–palladium, and viewed in a Hitachi S-3500 N scanning electron microscope.

Electron spin resonance (ESR) spectroscopy analyses. ESR spectroscopy has been used to study and define melanins based on the properties of unpaired electrons present in melanin (Enochs *et al.*, 1993) by using a Gunn diode as a microwave source. We used this method with a total of 2 g freeze-dried material in each case (analysis was carried out in silica cuvettes). *A. niger* melanin was used as a positive control.

Immunofluorescence analyses of melanin in *P. marneffei in vitro*. Immunofluorescence was performed on slide cultures of *P. marneffei* in the mycelial form. To examine yeast cells, paraffin-embedded yeast cells were cut into 4 μm sections, then deparaffined and rehydrated. Both sets of slides were washed three times with PBS, then incubated with Superblock Blocking Buffer in PBS (Pierce) for 2 h at 37 °C or overnight at 4 °C to block non-specific

binding.The slides were washed three times with PBS, then incubated for 1·5 h at 37 °C with 10 μg ml^{-1} of anti-melanin mAb 8D6 (against melanin of *A. fumigatus*; Youngchim *et al.*, 2004) made up in Superblock blocking buffer in PBS. For a negative control, conjugated goat anti-mouse IgM without primary mAb was used. After washing three times with PBS, slides were incubated with a 1 : 100 dilution of fluorescein-isothiocyanate-conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories) for 1·5 h at 37 °C. The slides were washed three times with PBS to eliminate unbound antibody and then mounted in 50 % (v/v) glycerol/50 % PBS. Coverslips were applied and slides were examined using a Zeiss microscope.

Detection of melanization of *P. marneffei in vivo* via immunofluorescence. Paraffin-embedded skin tissue from *P. marneffei*-infected patients was sectioned. The sections were deparaffined in xylene, rehydrated in an ethanol series, treated with 20 μg Proteinase K ml^{-1} (Roche) for 1 h at room temperature, and then heated in 10 mM citric acid in a microwave oven for 5 min. Slides were incubated with Superblock Blocking Buffer in PBS (Pierce) for 2 h, and, after washing, stained with mAb 8D6 as described above.

Experimental infection of mice with *P. marneffei* ATCC 200051. Five 6–8-week-old female BALB/c mice were inoculated with *P. marneffei* ATCC 200051 conidia by inhalation of 10^5–10^7 conidia in 30 μl 0·85 % sterile saline solution. Infected mice were housed in sterile conditions in the animal facility of the Audie L. Murphy memorial Veterans Hospital, San Antonio, TX, USA, as were non-infected control mice. After 8 weeks, the infected and the control mice were sacrificed; their bloods were taken and anti-melanin antibody (Ab) responses detected by ELISA.

For ELISA, a suspension of 50 μg melanin extracted from *P. marneffei* conidia in distilled water was plated in each well of a polystyrene 96-well ELISA plate (Corning Glass Works) and incubated overnight at room temperature until dry. The melanin was then heat-fixed to the polystyrene solid-phase support by incubating the plates at 60 °C for 30 min. Wells were blocked to prevent non-specific binding by adding 200 μl 5 % (w/v) bovine serum albumin (BSA) in PBS at 4 °C for overnight. The plates were washed three times with 0·1 % (v/v) Tween 20 in Tris-buffered saline. Sera (from *P. marneffei*-infected mice) were diluted in 1 % (w/v) BSA in PBS, added (100 μl) to the wells of the melanin-coated plates, and incubated for 1·5 h at 37 °C. After three washes, 100 μl of a 1 : 1000 dilution of peroxidase-conjugated goat anti-mouse IgM or goat anti-mouse IgG (Jackson Immunoresearch Laboratories) in PBS was added to the wells and incubated for 1·5 h at 37 °C. The plates were then washed, and 100 μl *o*-phenylenediamine in 0·01 M sodium citrate buffer (pH 5·0) per well was used as the enzyme substrate. Plates were incubated for 10 min in the dark, and the reaction was stopped with 100 μl 0·01 M H_2SO_4 per well. The solutions were transferred to fresh ELISA plates and the A_{490} values were measured with an ELISA plate reader (Microplate Reader 450/550, Bio-Rad). ELISA was performed in triplicate and included a negative control which consisted either of uncoated wells or of coated wells in which the mice sera were omitted.

Data are presented as the mean $A_{490} \pm$ SEM. A two-tailed unpaired Student's *t* test was used to test the statistical significance between two groups. $P < 0{\cdot}05$ was considered significant. All statistical calculations were performed by using the Prism program.

Detection of laccase activity via non-denaturating gel electrophoresis. The laccase activities of *P. marneffei* were detected by using 10 % PAGE, with a gel run of 30 V overnight under non-denaturing conditions. Cytoplasmic antigens from *P. marneffei* conidia were extracted by using a homogenizer (Biospec) with 5 mm glass ballotini (Gómez *et al.*, 2001). The homogenate was centrifuged at 10 000 ***g*** for 30 min to pellet any debris. The supernatant liquid was concentrated by Amicon concentrators (cut-off 5000 Da) or by centrifugal filtration. Protein was estimated by the Coomassie brilliant blue method (Read & Northcote, 1981). Commercially prepared laccase (from *Rhus vernificera*, activity 180 units per mg solid) was obtained from Sigma. *R. vernificera* laccase (40 μg) and 300 μg cytoplasmic yeast antigens of *P. marneffei* ATCC 200051, NCPF 4060 and NCPF 4062 were loaded onto gels; after electrophoresis, the gels were immersed in 1 mM L-DOPA in 0·1 M citric acid/0·2 M Na_2HPO_4 (pH 6·0) buffer for 6–8 h. As a control, each of the above samples was boiled in water for 5 min prior to loading onto the gel.

RESULTS

Melanization of *P. marneffei* ATCC 200051 conidia and yeast cells

Conidia of *P. marneffei* were visibly pigmented after growth on MEA for 7–10 days. When dislodged and collected by centrifugation, the conidia appeared pigmented (results not shown). After treatment of the conidia with enzymes, denaturant and hot acid, dark particles were recovered. SEM identified these particles as being of the same size and shape as the original intact conidia (Fig. 1a, b). TEM analysis of the dark post-treatment particles revealed a layer of electron-dense material enclosing a void (Fig. 1c). In contrast, Pracharktam *et al.* (1992) determined that untreated *P. marneffei* conidia show the typical intracellular conidial ultrastructure. *P. marneffei* yeast cells grown on

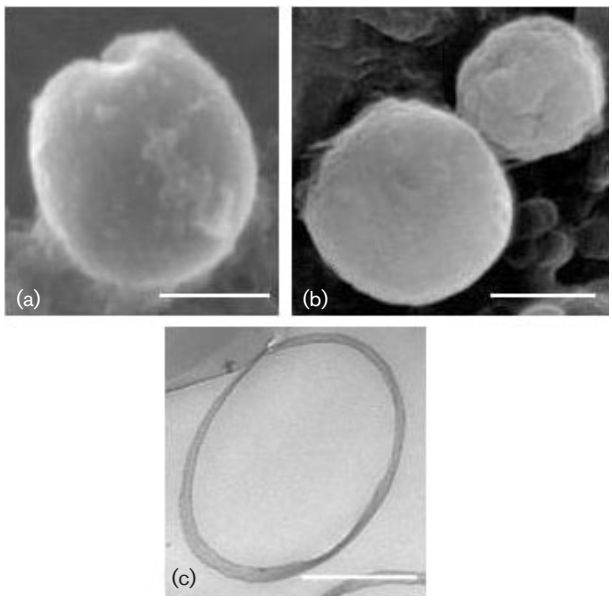

Fig. 1. (a, b) Scanning electron micrographs of *P. marneffei* ATCC 200051 before (a) and after (b) treatment with enzymes, denaturant and hot acid, which revealed particles (b) with the same size and shape as intact conidia. (c) Transmission electron micrograph of conidia after treatment with enzymes, denaturant and hot acid. Bars, 1 μm.

BHI broth at 37 °C produced dark brown pigment after 7 days, and black particles were isolated after treatment with melanin extraction reagents (data not shown).

ESR spectroscopy

ESR spectroscopy of dark particles extracted from *P. marneffei* ATCC 200051 conidia produced a signal which indicated that there was a stable free-radical population present identical to that which defines a pigment as melanin (Enochs *et al.*, 1993) (results not shown). The spectrum was virtually identical to the signal generated with melanins of *C. neoformans* (Wang & Casadevall, 1996), *Paracoccidioides brasiliensis* (Gómez *et al.*, 2001), *Histoplasma capsulatum* (Nosanchuk *et al.*, 2002) and *A. fumigatus* (Youngchim *et al.*, 2004).

Immunofluorescence analyses

The anti-melanin mAb 8D6 bound strongly to the cell walls of conidiophores and metulae during the conidiation of *P. marneffei* cultures (Fig. 2). Undifferentiated mycelial elements were unreactive with mAb 8D6 on slide culture. However, mAb 8D6 demonstrated immunoreactivity against melanin in the intact mycelia which had been sectioned prior to staining (data not shown). This may indicate poor penetration of mAb into the intact mycelia. Also, the mycelial culture of *P. marneffei* treated with enzymes, denaturant and hot acid resulted in dark particles. Additionally, yeast cells embedded in paraffin were reactive with mAb 8D6 (Fig. 3) and the melanin particles extracted from both conidia and yeast cells were also positively stained by mAb 8D6 (Fig. 4). There was no reactivity with conidia, yeast or melanin particles with the negative control, which involved the use of PBS in place of mAb 8D6 (data not shown).

The presence of yeast cells in issue sections of skin from patients infected with *P. marneffei* was confirmed by staining with haematoxylin/eosin and Gomori's methenamine silver (data not shown). Anti-melanin mAb exhibited reactivity with yeast cells present within monocytes in infected skin (Fig. 5).

Murine *P. marneffei* infection elicits antibodies to melanin

An ELISA for the detection of sera from mice infected with *P. marneffei* was developed using melanin particles extracted from *P. marneffei* conidia. Sera from these mice demonstrated a significantly higher titre of anti-melanin IgM and IgG against *P. marneffei* melanin when compared with uninfected mice ($P<0{\cdot}05$) (Fig. 6).

PAGE analysis of laccase-like activity

To determine whether *P. marneffei* had laccase activity, cytoplasmic antigens extracted from yeast cells of *P. marneffei* strains ATCC 200051, NCPF 4060 and NCPF 4062 were electrophoresed in a non-denaturing gel and then incubated

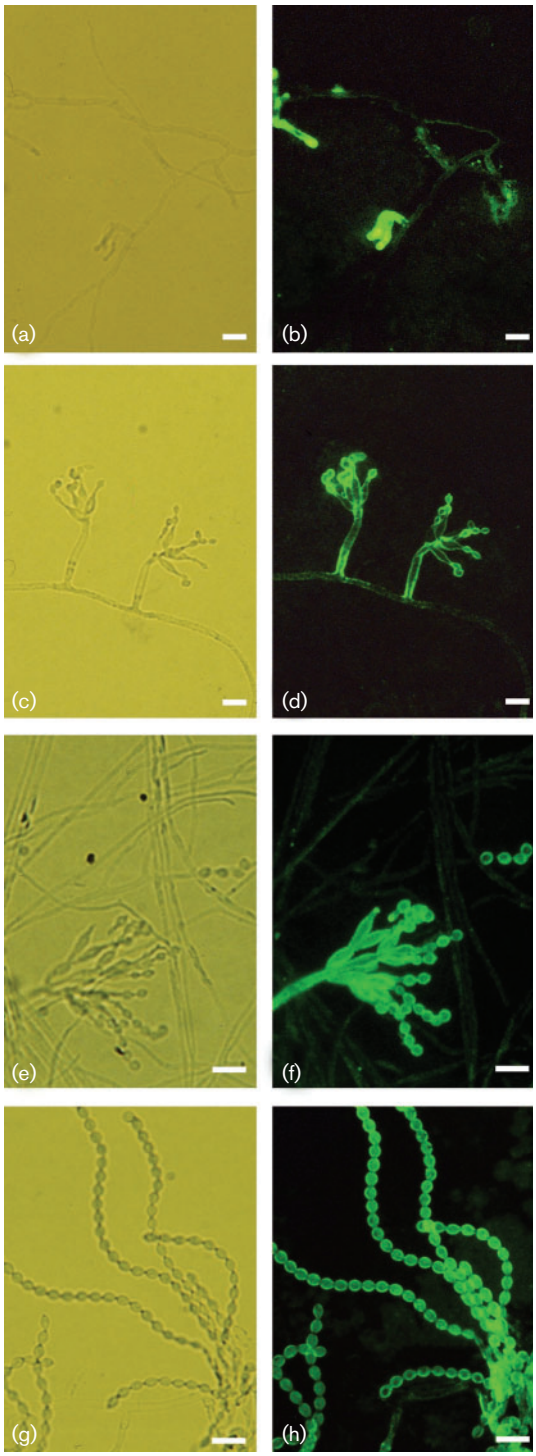

Fig. 2. Corresponding brightfield and immunofluorescence microscopy images demonstrating the labelling with melanin-binding mAb 8D6 of conidiophores, metulae and conidia during the conidiation of *P. marneffei* ATCC 200051: (a, b) 2 days old, (c, d) 4 days old, (e, f) 5 days old; (g, h) 7 days old. Bars, 5 μm.

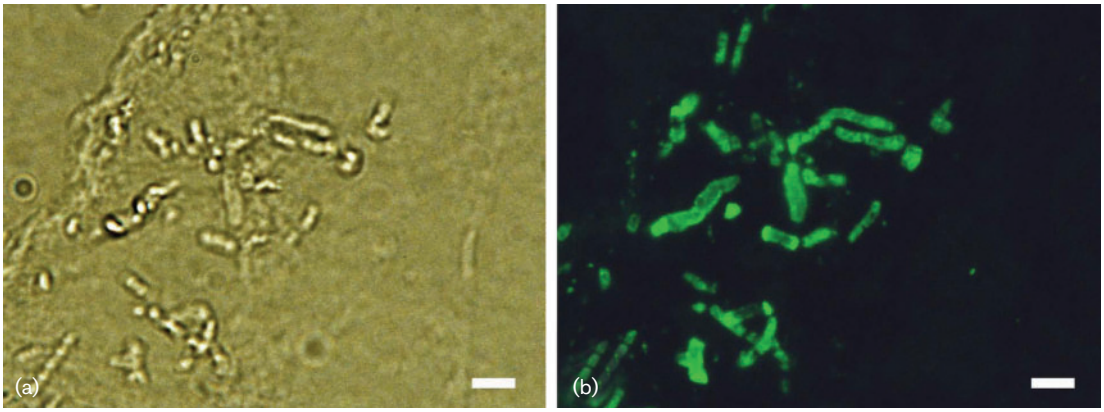

Fig. 3. Corresponding brightfield (a) and immunofluorescence (b) images showing the labelling of yeast cells of *P. marneffei* ATCC 200051 with melanin-binding mAb 8D6. Bars, 5 μm.

with L-DOPA for 6 h. This resulted in the formation of dark bands consistent with polymerized DOPA-melanin (results not shown). The same results were observed with the commercially available *R. vernificera* laccase. Boiling cytoplasmic antigens from *P. marneffei* and *R. vernificera* abrogated the enzymic activity.

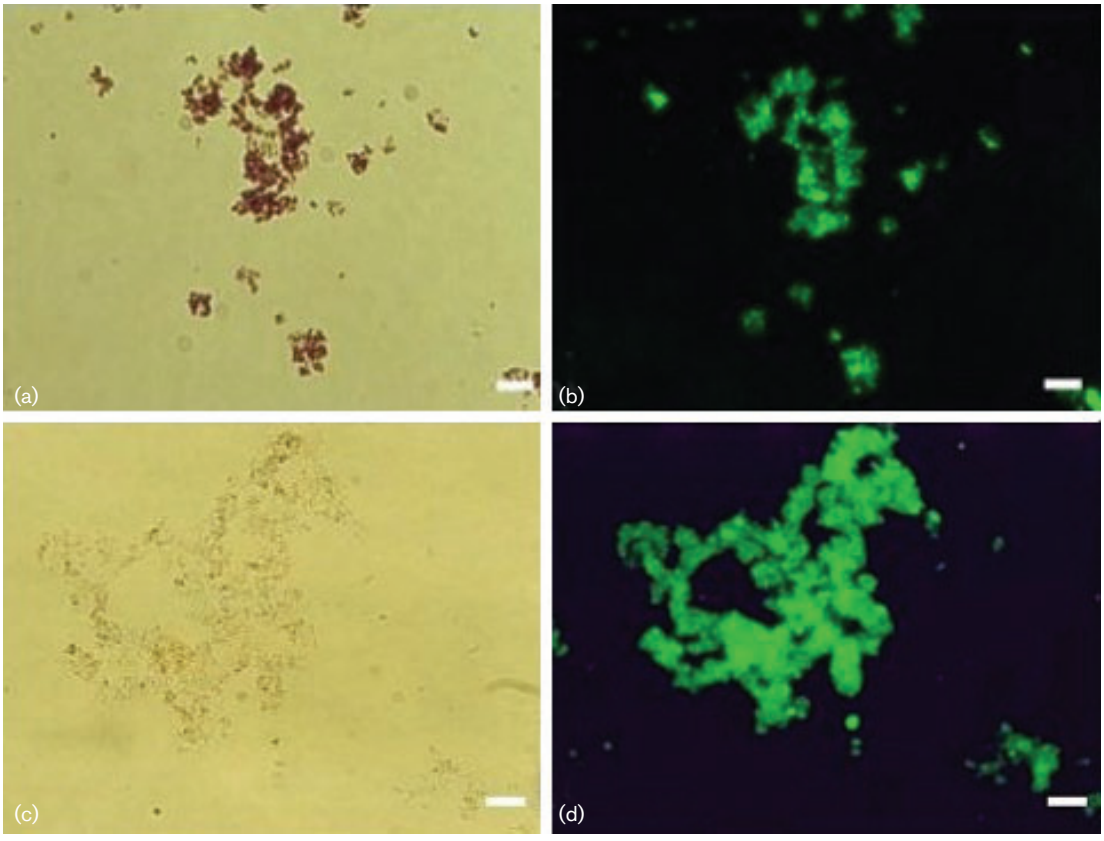

Fig. 4. Corresponding brightfield (a, c) and immunofluorescence (b, d) images showing the reaction of melanin particles extracted from conidia (a, b) and from yeast cells (c, d) of *P. marneffei* ATCC 200051 with melanin-binding mAb 8D6. Bars, 10 μm.

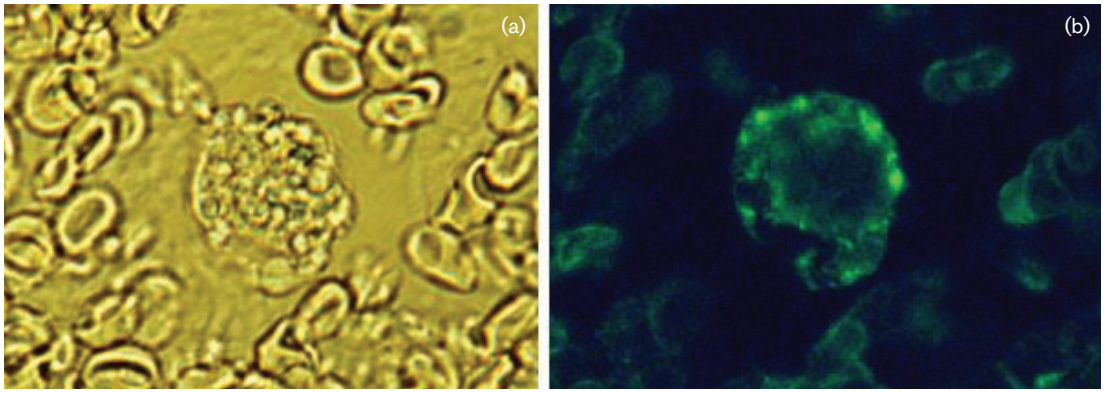

Fig. 5. Corresponding brightfield (a) and immunofluorescence (b) images demonstrating the labelling of *P. marneffei* yeast cells by melanin-binding mAb 8D6 in infected skin from a patient.

DISCUSSION

Diverse fungal pathogens have been shown to produce melanin-like pigments that are associated with virulence (Jacobson, 2000). We applied the techniques used for isolating melanin particles from other fungi, such as *C. neoformans*, *Par. brasiliensis* and *H. capsulatum* (Rosas *et al.*, 2000; Gómez *et al.*, 2001; Nosanchuk *et al.*, 2002), to investigate whether *P. marneffei* produces melanin. The evidence supporting the melanization of *P. marneffei in vitro* is as follows: (i) treatment of pigmented *P. marneffei* conidia with enzymes and chemicals resulted in the isolation of black particles similar in size and shape to their propagules, (ii) ESR spectroscopy analysis of black particles extracted from *P. marneffei* conidia indicated the presence of a stable free radical compound consistent with melanin, (iii) reactivity of a melanin-binding mAb to the cell wall of pigmented *P. marneffei* and pigmented particles derived from these cells, and (iv) detection of laccase activity in protein extracts of *P. marneffei* (both conidia and yeast). In combination, these observations provide good evidence that *P. marneffei* can produce melanin *in vitro*.

Analysis of melanization in *P. marneffei* using anti-melanin mAbs has shown that conidia and conidiophores express large amounts of melanin. These results are similar to the pattern of melanin synthesis seen in diverse fungal pathogens such as *Par. brasiliensis* (Gómez *et al.*, 2001), *H. capsulatum* (Nosanchuk *et al.*, 2002) and *S. schenckii* (Morris-Jones *et al.*, 2003). The ability of micro-organisms to synthesize melanin is likely to be associated with a survival advantage in the environment. For instance, the facultative melanotic fungus *C. neoformans* is able to produce melanin in its natural habitat (Nosanchuk *et al.*, 1999). Fungi with melanized conidia are more resistant than those with non-melanized conidia to killing by UV light (Durrell, 1964; Vasilevskaya *et al.*, 1970; Zhdanova *et al.*, 1973) or solar irradiation (Zhdanova *et al.*, 1981). Hence, conidial melanization in *P. marneffei* may protect against environmental insults. This observation is also important

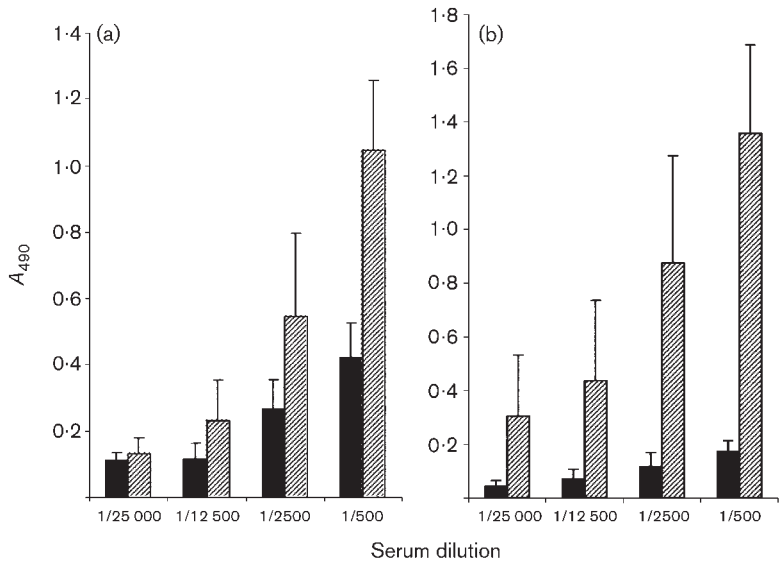

Fig. 6. Detection of anti-melanin IgM (a) and IgG (b) response by ELISA in *P. marneffei*-infected mice (hatched bars) compared with control (uninfected) mice (black bars). Each bar represents the mean ± SEM value for five infected mice. $P < 0{\cdot}05$ was considered significant when compared with control (uninfected) mice.

in terms of pathogenesis because it indicates that initial infection occurs with melanized propagules.

Infection with *P. marneffei* is presumed to occur after the inhalation of airborne conidia, i.e. it involves a respiratory route of infection. An association between the presence of melanin and protection against the mammalian immune response has been studied in several pathogenic fungi including *A. fumigatus* (Brakhage *et al.*, 1999; Jahn *et al.*, 2000) and *C. neoformans* (Kwon-Chung & Rhodes, 1986; Wang *et al.*, 1995). In the study described here, mice infected with *P. marneffei* developed a significant antibody response against melanin, which implies that melanin can act as an immunologically active molecule. In this respect, this study is similar to the published work on melanization in *H. capsulatum* during murine infection, which demonstrated that sera from infected mice contained antibodies that bound melanin particles (Nosanchuk *et al.*, 2002). Development of an antibody response to melanin during murine cryptococcosis has also been noted (Nosanchuk *et al.*, 1998).

It is perhaps unsurprising that *P. marneffei* yeast cells were shown to be pigmented when cultured on BHI medium, given that this medium is prepared from bovine tissues which are rich sources of phenolic compounds – substrates which may be associated with the production of melanin. Similarly, *S. schenckii* yeast cells have been shown to produce melanin when grown on BHI agar (Morris-Jones *et al.*, 2003) whilst *C. neoformans* can synthesize melanin when grown on minimal medium agar supplemented with homogenates of murine brains and lungs (Rosas *et al.*, 2000). These findings suggest that these phenolic compounds may be sufficient precursors to support the melanization of *P. marneffei* under these *in vitro* conditions. Furthermore, as phenolic compounds have been shown to be present in tissue (Rosas *et al.*, 2000), they may well be precursors in the biochemical production of melanin by laccase in *P. marneffei* yeast cells *in vivo*.

The detection of laccase-like activity in non-denaturing protein gels is consistent with the presence of such an enzyme in *P. marneffei*, indicating that *P. marneffei* has the enzymic machinery to synthesize melanin. Similarly, the presence of laccase-like activity was detected in cytoplasmic antigens of *C. neoformans* (Wang *et al.*, 1995), *Par. brasiliensis* (Gómez *et al.*, 2001) and *H. capsulatum* (Nosanchuk *et al.*, 2002). Ikeda *et al.* (2002) also reported a correlation between melanin synthesis and intracellular laccase in *C. neoformans*. Identification and the subsequent generation of mutants lacking this laccase-like enzyme in *P. marneffei* yeast cells are fundamental to determining the putative role of the enzyme in this biochemical pathway.

Our TEM study of *P. marneffei* conidia indicates that melanin is deposited primarily in the cell wall of conidia; as such it is in a position in which it may be recognized by immune cells. The biological function of antibodies to fungal melanin is unknown but the fact that melanin is accessible to antibody binding raises the possibility that melanin-binding antibody could mediate biological effects during *P. marneffei* infection. A key finding is that an anti-melanin mAb demonstrated reactivity to yeast cells from infected skin tissue in penicilliosis marneffei patients, indicating that *P. marneffei* yeast cells synthesize melanin-like pigments during mammalian infection. These results are similar to those of studies showing that *C. neoformans* (Nosanchuk *et al.*, 2000; Rosas *et al.*, 2000), *Par. brasiliensis* (Gómez *et al.*, 2001), *H. capsulatum* (Nosanchuk *et al.*, 2002) and *S. schenckii* (Morris-Jones *et al.*, 2003) yeast cells are melanized *in vivo*.

In conclusion, our results demonstrate that *P. marneffei* conidia and yeast cells can synthesize melanin or melanin-like pigments when grown *in vitro* and have also shown that a melanin-binding mAb labelled yeast cells in infected tissues. Additionally, an antibody response against melanin has been detected in *P. marneffei*-infected mice, which implies that the cells synthesized melanin or melanin-like pigments during infection. In this respect, melanin presumably contributes to *P. marneffei* virulence by promoting survival within host tissue. Further studies should concentrate on the generation of melanin-deficient mutants in order to examine their virulence relative to melanized cells.

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