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Scytalidium dimidiatum Causing Recalcitrant Subcutaneous Lesions Produces Melanin

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Scytalidium dimidiatum **is a pigmented dematiaceous coelomycete that typically causes chronic superficial skin diseases and onychomycosis, as well as deeper infections, such as subcutaneous abscesses, mycetoma, and even fungemia in immunocompromised patients. A second species,** ***Scytalidium hyalinum*****, has hyaline hyphae and arthroconidia and is considered by some authors to be an albino mutant of** ***S. dimidiatum*****. This study aimed to confirm the presence of melanin or melanin-like compounds (which have been previously implicated in the virulence of other fungal pathogens) in** ***S. dimidiatum*** **from a patient with multiple subcutaneous nodules. Treatment of the hyphae and arthroconidia with proteolytic enzymes, denaturant, and concentrated hot acid yielded dark particles, which were stable free radicals, consistent with their identification as melanins. Extracted melanin particles from** ***S. dimidiatum*** **cultures were labeled by melanin-binding monoclonal antibodies (MAbs) from** ***Sporothrix schenckii*****,** ***Aspergillus fumigatus*****, and** ***Cryptococcus neoformans*****. Lesional skin from the patient infected with** ***S. dimidiatum*** **contained fungal cells that were labeled by melanin-binding MAbs, and digestion of the tissue yielded dark particles that were also reactive.** ***S. hyalinum*** **was also subjected to the melanin extraction protocol, but no dark particles were yielded.**

Scytalidium dimidiatum (synanamorph, *Nattrassia mangiferae*, formerly known as *Hendersonula toruloidea*) is a thermotolerant fungal plant pathogen that can cause superficial skin and nail infections that mimic those caused by dermatophytes (7). *S. dimidiatum* can also rarely cause deep subcutaneous infections and disseminated disease, mainly in immunosuppressed patients (2, 17, 31). Subclinical infection is thought to be common in parts of Africa, India, and the Caribbean, where the fungus lives in the soil and on plants (1, 11, 23). *S. dimidiatum* forms a rapidly growing brown-black colony, which is cycloheximide sensitive (11).

Melanins are enigmatic high-molecular-weight pigments that are produced by a wide variety of microorganisms. They are typically dark brown or black and are insoluble in aqueous and organic solvents and are therefore difficult to study by conventional biochemical techniques (9). Melanin has been isolated from several important human fungal pathogens and is now recognized as an important virulence determinant (21).

In addition, a second species, *Scytalidium hyalinum*, has also been isolated from human sources (16), although there has been considerable debate in the literature as to whether *S. dimidiatum* and *S. hyalinum* are the same or distinct species. Some morphological and genetic characteristics (such as mycelium pigmentation, intronic insertions in the 18S gene, and the A-G polymorphism [16]), apparently do differentiate these species. In contrast, Roeijmans et al. considered *S. hyalinum* to be a synonym of *S. dimidiatum* after conducting molecular taxonomy studies using restriction fragment length polymorphisms (26). Indeed, most authors agree that *S. hyalinum* may be a melanin-deficient cultural mutant of *S. dimidiatum* (18, 25, 32).

S. dimidiatum was isolated from a renal transplant patient with recalcitrant cutaneous lesions (J. M. Hextall, D. M. MacDonald, J. E. Scoble, and R. J. Hay, abstract from Br. Assoc. Dermatol. Annu. Meet., Br. J. Dermatol. **145:**34, 2001). Mycological culture of this isolate grown on Sabouraud dextrose agar revealed a black colony. We were able to extract melanin-like particles for the first time from this human pathogenic fungus, using enzymes, denaturant, and hot acid. Melanin has been implicated in the pathogenesis of fungal infections and may therefore play a role in the recalcitrant nature of *Scytalidium* infections in humans.

CASE REPORT

This clinical case was described previously (Hextall et al., Br. J. Dermatol. **145:**34, 2001). Briefly, a 66-year-old man with a history of diabetes and renal transplantation presented with violaceous nodular lesions on the left lower leg. The histopathology from a biopsied nodule showed suppurative granulomas in the dermis and numerous fungal elements, which stained positive with methenamine silver (Grocott modification) (Fig. 1). Culture and microscopy grew a grey-brown mould, which had the typical morphology of *S. dimidiatum*. The patient was treated with intravenous liposomal amphotericin B (Ambisome) for 14 days; the lesions eventually regressed, and the patient was discharged on oral terbinafine to continue long-term therapy.

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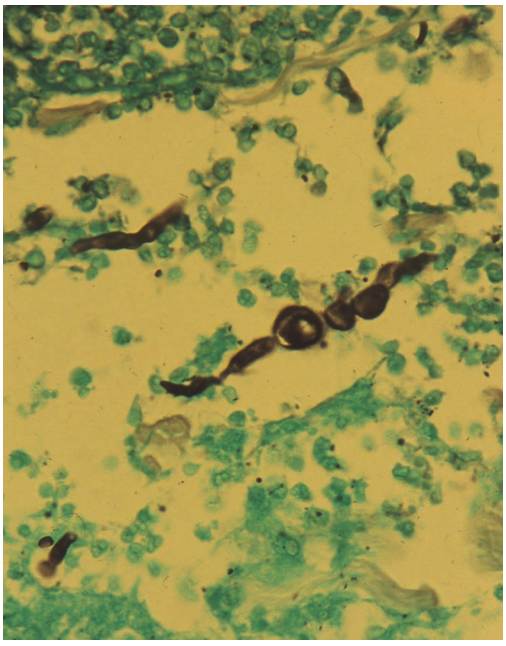

FIG. 1. Grocott stain showing *S. dimidiatum* arthroconidia and hyphae in the dermis. Magnification, ×64.

MATERIALS AND METHODS

Fungal strains and media. *S. dimidiatum* (strain 10810) from the index case was obtained from one of the authors (S. D. Morris-Jones). *S. hyalinum* (strain 2775) was obtained from the National Collection of Pathogenic Fungi in Bristol, United Kingdom.

Isolation and purification of *S. dimidiatum* hyphal and arthroconidial particles, scanning electron microscopy, transmission electron microscopy, and ESR. Melanin particles were isolated from pigmented hyphae and arthroconidia grown for 1 week on Sabouraud dextrose agar (SAB) and minimal medium (10, 30). In brief, cells were collected and washed three times with phosphate-buffered saline (0.1 M; pH 7.5) and suspended in 1.0 M sorbitol–0.1 M sodium citrate (pH 5.5). The cells were treated in turn with lysing enzymes (from *Trichoderma harzianum*; Sigma Poole, Dorset, United Kingdom), 4 M guanidine thiocyanate (denaturant), and proteinase K (Roche Laboratories, Lewes, Sussex, United Kingdom). The resultant material was then boiled in 6 M HCl, washed, and collected. Scanning and transmission electron microscopy and electron spin resonance (ESR) spectroscopy (using a Gunn diode as the microwave source) analyses of melanin particles were then performed, as previously described (4, 30).

Immunofluorescence analysis of melanin expression. Melanin particles derived from *S. dimidiatum* were fixed to slides and blocked with Superblock (Roche) overnight at 4°C. Slide cultures of *S. dimidiatum* and *S. hyalinum* were prepared as described previously (19) and blocked as described above. All slides were then incubated for 2 h at 37°C with 10 μg of either the anti-*Sporothrix schenckii* melanin monoclonal antibody (MAb) 8B5 or the melanin-binding MAb 6D2. The anti-*S. schenckii* melanin MAb 8B5 had been previously generated against *S. schenckii* yeast cell melanin particles (19), and MAb 6D2 was raised against melanin from *Cryptococcus neoformans* (29). The slides were washed in phosphate-buffered saline, incubated in a 1:100 dilution of fluorescein isothiocyanate (FITC) GAM-immunoglobulin M (IgM) (Stratech Scientific, West Grove, Pa.) for 2 h at 37°C, and then washed again. Negative controls consisted of either the irrelevant antibody 5C11 (IgM), which binds to lipoarabinomannan of mycobacteria (8), as the primary antibody or FITC-labeled antibody alone. To examine in vivo expression of *S. dimidiatum* melanin, paraffin-embedded samples from lesional skin of the patient were sectioned. The sections were deparaffinized, rehydrated, treated with 20 μg of proteinase K/ml for 1 h at 21°C, and then heated in citric acid in a microwave for 5 min. The slides were blocked and labeled as for the in vitro work with the same negative controls described above. The paraffin-embedded sections from the patient were also subjected to the melanin extraction protocol. Normal human skin was used as a negative control. *S. dimidiatum* melanin particles were isolated from the infected tissue and air dried on APES (3-aminopropyltriethoxysilane)-covered slides. The slides were then probed with the various anti-melanin MAbs and FITC GAM-IgM as described above. Wild-type *S. schenckii* (Mel^+) and its albino mutant (Mel^-) were used as positive and negative controls (19). The wild-type *C. neoformans* JEC21 grown in the presence of L-3,4-dihydroxyphenylalanine produces melanin and was also used as a positive control, and the *C. neoformans* albino mutant MHC6 (melanin deficient) was used as an additional negative control (29).

RESULTS

Melanization of *S. dimidiatum* hyphae and arthroconidia. Mycelia of isolate 10810 were visibly pigmented when grown on minimal medium and Sabouraud dextrose agar for 3 days. Black particles that were of a shape and size similar to the original propagules (hyphae and arthroconidia) were isolated from *S. dimidiatum* cultures that were subjected to the extraction protocol, as demonstrated by scanning electron microscopy (Fig. 2). Transmission electron microscopy of *S. dimidiatum* hyphae and arthroconidia extracted with enzymes, denaturant, and hot acid (Fig. 3) showed a layer of electron-dense granules enclosing a void. Mycelia of *S. hyalinum* (strain 2775) were white when grown on minimal medium or Sabouraud dextrose agar. The hyaline arthroconidia and hyphae of *S. hyalinum* were also subjected to the melanin extraction protocol, on completion of which no particles were retrieved

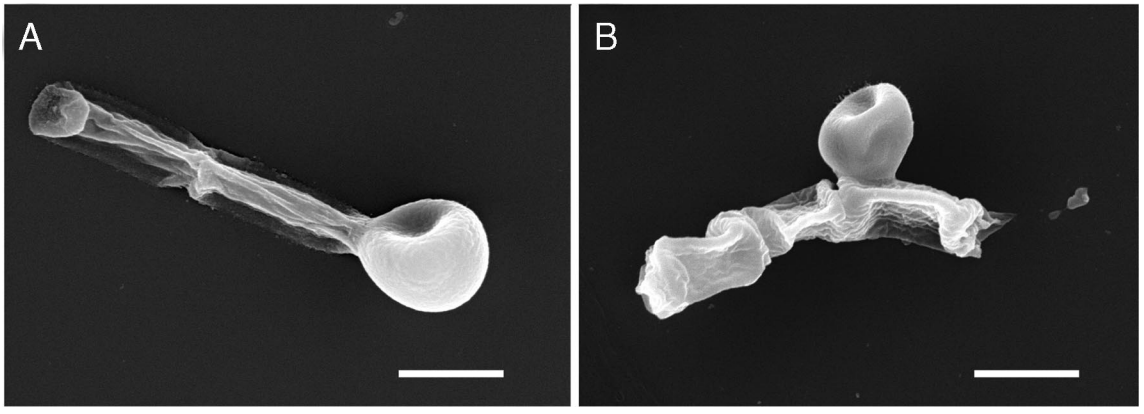

FIG. 2. Scanning electron microscopy of *S. dimidiatum* hyphae and arthroconidia before (A) and after (B) treatment with enzymes, denaturant, and hot acid. (A) *S. dimidiatum* culture. (B) Extracted melanin particles. Bars, 5 μm.

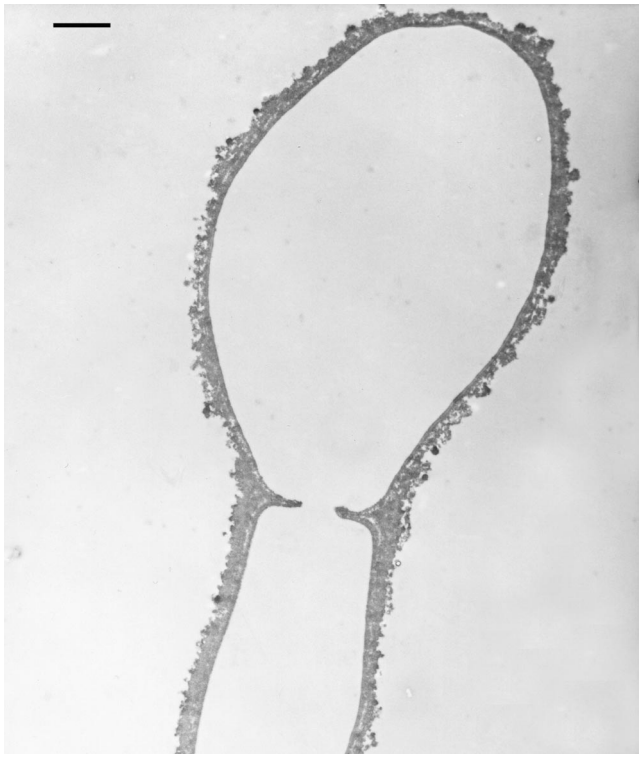

FIG. 3. Transmission electron micrograph of *S. dimidiatum* ghost showing outer melanin layers. Bar, 0.5 μm.

from the 6 M HCl, as the fungus had been completely solubilized.

ESR spectroscopy. ESR spectroscopy of the melanin-like particles collected from *S. dimidiatum* produced a stable free-radical population consistent with the pigment being melanin (Fig. 4). The spectrum was virtually identical to the signals generated by melanin previously extracted from *C. neoformans* (35), *Paracoccidioides brasiliensis* (10), and *Histoplasma capsulatum* (22).

Immunofluorescence analysis. The anti-*S. schenckii* melanin MAb 8B5 bound to the surfaces of the pigmented hyphae and arthroconidia (Fig. 5A and B), as did MAb 6D2. Melanin-like particles extracted from mycelia were reactive to the anti-melanin MAb 8B5 (Fig. 5C and D). Neither of the anti-melanin MAbs bound to the arthroconidia and hyphae of *S. hyalinum*.

Isolation of melanin particles from infected tissue. Tissue sections from the skin of the index case patient infected with *S. dimidiatum* were stained with methenamine silver (Grocott modification) to confirm the presence of fungus (Fig. 1). Anti-melanin MAbs demonstrated reactivity to *S. dimidiatum* cells in infected tissue (Fig. 6 A and B), which was absent when FITC-labeled GAM-IgM was used alone or when isotype-matched negative control MAb 5C11 (IgM) was used. Treatment of infected human tissue using the melanin extraction protocol resulted in the isolation of dark particles. No particles resembling *S. dimidiatum* were isolated from normal human skin. The pigmented particles reacted with anti-melanin MAbs in a manner similar to the in vitro-isolated particles (Fig. 6C and D).

DISCUSSION

S. dimidiatum lives on the roots of certain plants, mainly of the genera *Plantus* and *Pinus* (14). It is relatively thermotolerant and can cause significant plant pathology, especially of fruit trees. Since the first report of human disease caused by *H. toruloides* (*S. dimidiatum*) (7), there have been numerous case reports from areas of endemicity. In humans, *S. dimidiatum* is associated with sporadic, as well as epidemic, disease. *S. dimidiatum* most commonly causes chronic cutaneous infections, but it has been reported to cause mycetoma, subcutaneous abscesses, fungemia, and endophthalmitis following trauma (31). . Two previous cases of *S. dimidiatum* causing subcutaneous abscesses were reported in patients with underlying diabetes (3; D. A. McGough, C. R. Bodem, K. Fawcett, P. Moody, A. W. Fothergill, and M. G. Rinaldi, Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992, abstr. F-26, p. 503). More recently, cases have been reported in immunosuppressed patients with AIDS (17).

The first description of *S. dimidiatum* (32) documented its tendency to form colonies with dark pigmentation. Subsequently, there have been numerous reports of a white, pigment-deficient variant, namely, *S. hyalinum*, being isolated from human infections (19). Recent work has suggested that members of the genus *Scytalidium* may synthesize melanin—thus, *Scytalidium* species produce scytalols A to D, which are modulators of melanin biosynthesis (5, 33). In addition, the enzyme laccase (which is involved in melanization [30]) has been isolated from *Scytalidium thermophilum* (39). However, no comprehensive study has been done to confirm the presence of melanin, despite the potentially important role that the pigment may play in pathogenesis. There is an increasing body of evidence that melanization prolongs the survival of fungi in the environment (9). Thus, *C. neoformans* is protected from environmental insults, such as UV radiation, extremes of temperature, and oxygen and nitrogen free radicals, by melanin in its cell wall (27, 37, 38). Interestingly, *S. dimidiatum*, which is pigmented, is found in the environment, whereas the nonpigmented *S. hyalinum* has been found only in humans and never in the environment.

In the present study, we have confirmed experimentally that *S. dimidiatum* does indeed produce melanin-like pigment. Our evidence for this is as follows: (i) treatment of *S. dimidiatum* hyphae and arthroconidia with enzymes and chemicals resulted in the isolation of black particles that were of a shape and size

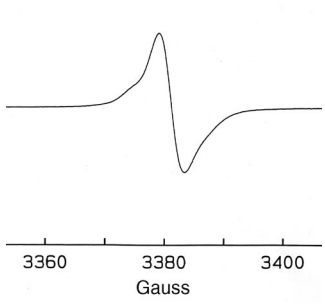

FIG. 4. ESR spectroscopy of melanin particles collected from *S. dimidiatum* grown for 7 days in Sabouraud dextrose agar.

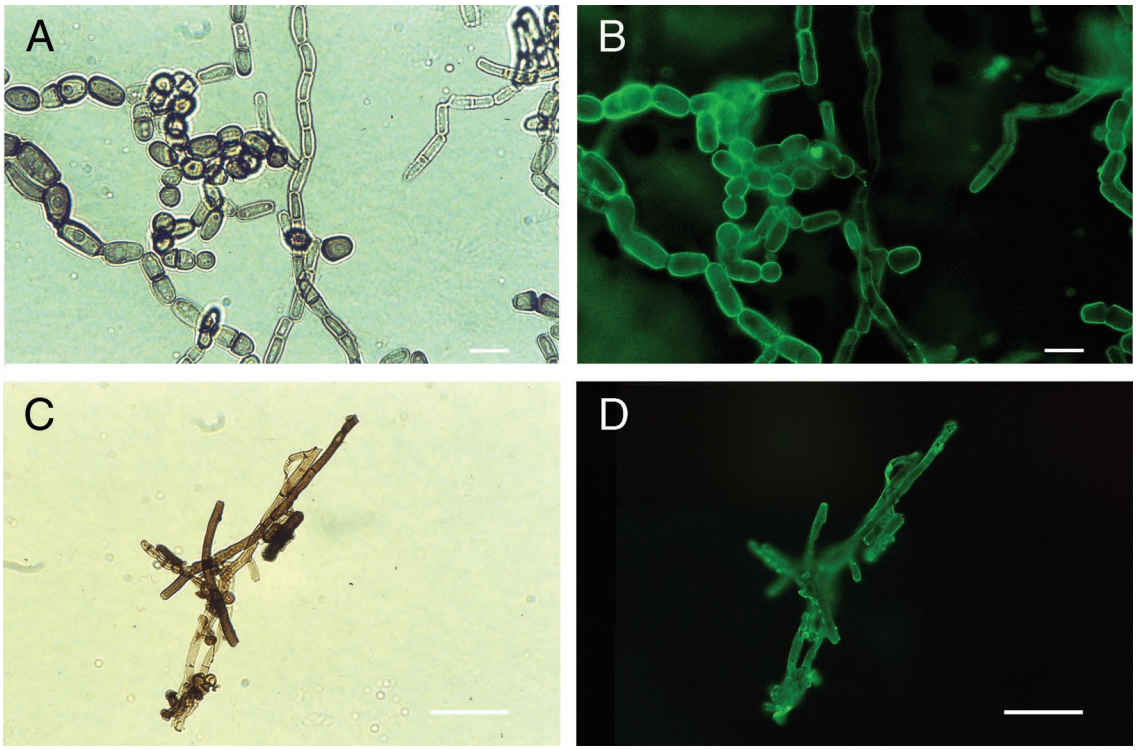

FIG. 5. Corresponding bright-field (A) and immunofluorescence (B) microscopic images of *S. dimidiatum* from slide culture (in vitro) were reacted with anti-*S. schenckii* melanin MAb 8B5. Corresponding bright-field (C) and immunofluorescence (D) images of melanin particles extracted from *S. dimidiatum* preparations reacted with anti-*S. schenckii* melanin MAb 8B5. Bars, 10 μm.

similar to the original propagules; (ii) ESR spectroscopy analysis of pigmented particles showed the presence of a stable free-radical compound consistent with melanin; (iii) melanin-binding MAbs reacted with the cell surface of *S. dimidiatum* grown in vitro and with the pigmented particles derived from these cells, and hyphal structures were melanized, which is in contrast to *H. capsulatum* (22) and *P. brasiliensis* (10); (iv) melanin-binding MAbs reacted with the cell wall of *S. dimidiatum* in infected human skin; and (v) we recovered melanin-like particles that were similar in shape and size to *S. dimidiatum* from infected human tissue after enzymatic and chemical treatment and which were also reactive with the melanin-binding MAbs. Together these observations provide good evidence that *S. dimidiatum* can produce melanin in vitro and in vivo. We have also confirmed for the first time that *S. hyalinum* is unable to produce melanin in vitro.

There is evidence from studies of *C. neoformans* that melanin synthesis is associated with increased virulence (20, 28). This finding might lead us to expect only *S. dimidiatum* to be isolated from patients; however, the melanin-deficient *S. hyalinum* has also been found in vivo. This observation may initially seem contradictory to the view that fungal melanization plays a role in virulence. However, the published data pertaining to the *Scytalidium* species is somewhat fragmentary. In fact, *S. dimidiatum* and *S. hyalinum* can be singularly and concurrently isolated by mycological culture from individual patients (1, 12, 23). Given that *S. hyalinum* has not been isolated from the external environment, this strongly suggests that the latter must arise in vivo from the black *S. dimidiatum*, possibly through phenotypic switching, for which there is evidence from other pathogenic fungi (6, 15). In keeping with this is evidence from ribosomal gene studies that has confirmed that *S. hyalinum* is genetically indistinguishable from *S. dimidiatum* (26). Further investigations are needed to explore the enzymes and genetic components of *S. hyalinum* to see if it has the necessary machinery to synthesize melanin. From this information, we may be able to identify possible triggers for the production and degradation of melanin in the environment and in vivo.

S. dimidiatum infections remain highly resistant to the available antifungal drugs, despite adequate drug levels in serum and tissue (determined from MICs) (11). The reasons for this are not yet known; however, melanin has been shown to be important in resistance to antifungal drugs in *C. neoformans* (13, 36) and *H. capsulatum* (34). It is thus possible that melanization in *S. dimidiatum* may also play a role in drug resistance. Clinically, however, it appears that the white and the black isolates of *Scytalidium* are equally resistant to antifungals. This observation is in keeping with studies of *Wangiella dermatitidis*, in which loss of melanin did not increase susceptibility to antifungal agents (24). Our patient did not respond to treatment with itraconazole, and clinical improvement was evident only after a prolonged course of liposomal amphotericin B. His toenail clippings remained culture positive for *Scytalidium* up to 1 year after this treatment. The patient continues on the immunosuppressant drugs needed to protect his renal allograft, and therefore long-term secondary prophylaxis

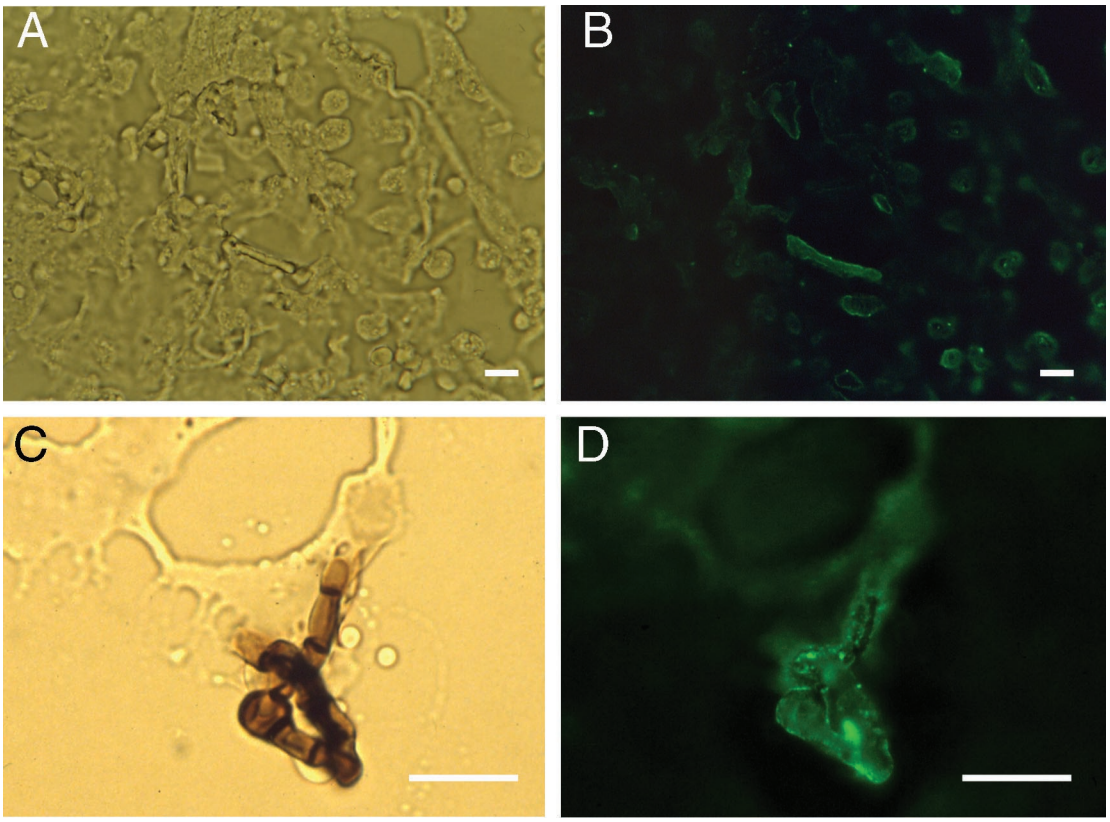

FIG. 6. Corresponding bright-field (A) and immunofluorescence (B) microscopic images of human skin (in vivo) showing labeling of *S. dimidiatum* by MAb 8B5 (anti-*S. schenckii* melanin MAb). Corresponding bright-field (C) and immunofluorescence (D) images showing melanin particles recovered from human skin also labeled with MAb 8B5. Bars, 5 μm.

with terbinafine may be needed to prevent relapse of the cutaneous *Scytalidium* infection.

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