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Development of Specific Fluorescent-Antibody Test for Tissue Form of *Penicillium marneffei*

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The diagnosis of penicilliosis marneffei can be difficult because the clinical manifestations mimic those of tuberculosis, histoplasmosis, and other mycotic infections. Furthermore, the tissue form of *Penicillium marneffei* can be confused with those of *Histoplasma capsulatum* and *Cryptococcus neoformans*. To facilitate the rapid detection and identification of *P. marneffei* in clinical materials, we sought to develop a specific indirect fluorescent-antibody (IFA) reagent for this dimorphic pathogen. Preliminary IFA studies with yeast-like cells (fission arthroconidia) of *P. marneffei* indicated that these cellular elements stained with antiglobulins against culture filtrate antigens and whole yeast-like cellular antigens. Both types of antiglobulins reacted with the yeast-like cells of *P. marneffei* and with *H. capsulatum*, but not with their respective mycelial forms. The antiglobulins also failed to react with the yeast and hyphal forms of a variety of other heterologous fungi. Specific antiglobulins useful in an IFA test for identifying *P. marneffei* yeast-like cells in culture or in clinical materials were produced by adsorptions with yeast-form cells of *H. capsulatum*. The yeast-like culture filtrate antigens of *P. marneffei* are preferred for use in the production of the specific antiglobulins because they stained *P. marneffei* yeast-like elements more intensely than antiglobulins produced against intact yeast-like cells.

Penicillium marneffei is an emerging opportunistic fungal pathogen among human immunodeficiency virus-infected and other immunocompromised humans who have traveled or resided in Southeast Asia (1). At 25°C the fungus grows as a mold and produces a deep, wine red, water-soluble pigment that diffuses into the agar medium, whereas at 37°C in vitro or in vivo it converts to a yeast-like form, displaying spherical or ellipsoidal cells measuring 2 to 3 by 2 to 6 μm which multiply by fission rather than budding. It is the only known *Penicillium* species that is dimorphic. The diagnosis of penicilliosis marneffei can be troublesome because its clinical manifestations mimic those of tuberculosis, pneumocystosis, histoplasmosis, and other mycotic infections. Furthermore, in vivo the fission arthroconidia of *P. marneffei* can be confused with those of nonbudding *Cryptococcus neoformans* and *Histoplasma capsulatum* cells. The study described here was undertaken to develop a specific indirect fluorescent-antibody (IFA) reagent for the rapid identification of *P. marneffei* in histologic sections.

MATERIALS AND METHODS

Fungal strains and antigen production. Stock cultures of three *P. marneffei* strains, strains ATCC 18224, ATCC 64102, and ATCC 24100, were maintained on Sabouraud dextrose agar slants at 25°C and on agar slants described by Pine (5) at 37°C. Preliminary studies were carried out with all three strains grown in brain heart infusion broth (BHIB), Pine's broth (PB) (5), Sabauraud dextrose broth (SDB), and Smith's synthetic asparagine broth (SAB) at 25 and 37°C. Six 1-liter flasks containing 500 ml of each medium were prepared. Three sets of duplicate flasks containing each medium were inoculated with 1.0 ml of a suspension of 5- to 7-day-old fission arthroconidia of each of the *P. marneffei* strains; the suspensions were adjusted turbidimetrically to a McFarland no. 8 nephelometer standard. One of each duplicate set of broth cultures was incubated while shaking at 25°C, and the other set was incubated at 37°C in a gyratory shaker-incubator. Cultures were grown for 1 month and were checked weekly, after removal of 50-ml aliquots, for the degree of turbidity and microscopic morphology. Cultures were rendered sterile by adding Merthiolate to a concentration of 0.02%. After appropriate sterility checks, the cells were removed by centrifugation, and the supernatants were concentrated 10 times by ultrafiltration through a 10,000-Da-exclusion-limit PM-10 membrane filter (Amicon Corp., Lexington, Mass.) and were retained.

At 25°C, filamentous growth ranged from scanty in SAB to moderate in BHIB, SDB, and PB after 2 weeks of incubation. At 37°C all strains exhibited poor growth in SAB and moderate growth with partial conversion to the yeast-like form in BHIB and SDB after 2 weeks of incubation. All three strains exhibited heavy growth and complete conversion after 2 weeks in PB. Exoantigen tests performed with the 37°C culture filtrates as described by Sekhon et al. (6) indicated that more and higher-titer precipitinogens occurred in PB than in SDB and BHIB. Furthermore, the 37°C culture filtrates contained more and higher-titer precipitinogens than the 25°C culture filtrates. On the basis of these preliminary studies, the 2-week-old fission arthroconidial cellular and filtrate preparations of strain ATCC 18224 grown in PB were chosen for use in all subsequent studies.

Antisera and antiglobulins. Large (weight, 4 kg) female New Zealand White rabbits were immunized with whole fission arthroconidia and culture filtrates of the type strain (ATCC 18224) of *P. marneffei*. Whole fission-form cell suspensions of *P. marneffei* grown in PB for 2 weeks at 37°C were washed three times with phosphate-buffered saline (PBS; pH 7.2) containing 0.02% Merthiolate. For immunizations the cells were resuspended in PBS and were adjusted to a turbidity equal to a McFarland no. 8 nephelometer standard. Doses of 2, 2, and 1 ml, respectively, were injected intravenously into two rabbits on alternate days for the first week. Two and 3 weeks later, each rabbit received three 1-ml intravenous injections on alternate days. Antisera against the filtrate antigens were prepared by injecting two rabbits subcutaneously in the dorsal area with an emulsion consisting of 1.0 ml of 10×-concentrated PB filtrate and 1.0 ml of Freund's incomplete adjuvant. Three weeks after the initial injection, the rabbits were subcutaneously injected with a another 1.0-ml dose of the filtrate-Freund's incomplete adjuvant emulsion. Two weeks after the second injection the rabbits were injected intravenously with 0.5 ml of culture filtrate antigen.

One week after the final injection, each rabbit was bled and its serum was tested for antibody against *P. marneffei* filtrate antigen in the immunodiffusion (ID) test. Sera produced against the intact yeast-like form produced one broad band, whereas sera produced against the culture filtrate produced one to five bands. The antiglobulin fractions were obtained from each antiserum by three successive precipitations with 35% saturated ammonium sulfate.

ID procedure. ID tests for antibodies and antigens were performed as described by Sekhon et al. (6).

Simulated tissues. Mock tissue sections consisting of finely minced human kidney tissue pellets were admixed with small numbers of tissue-form *P. marneffei* by the modified method of Swisher and Nicholson (7). Specimens were processed for 16 h in a tissue processor (Fisher Histomatic; Fisher Scientific, Pittsburgh, Pa.), embedded in Polyfin (Triangle Biomedical Sciences, Durham, N.C.) em-

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TABLE 1. IFA staining reactions of unadsorbed rabbit fission-form *P. marneffei* antiglobulins to culture filtrate and whole-cell antigens with homologous and heterologous dimorphic and other fungi

Fungus species (no. of strains)	Form[a]	Fluorescence for antiglobulin to[b]:	
		Filtrate antigens	Whole-cell antigens
Penicillium marneffei (3)	Y	3–4+	2–3+
Penicillium marneffei (5)	M	0	0
Penicillium spp. saprophytes (5)	M	0	0
Aspergillus flavus (2)	M	0	0
Aspergillus fumigatus (2)	M	0	0
Candida albicans (3)	Y	0	0
Candida glabrata (2)	Y	0	0
Cryptococcus neoformans (2)	Y	0	0
Histoplasma capsulatum (5)	Y	2–4+	2–3+
Histoplasma capsulatum (2)	M	0	0

[a] Y, yeast or yeast-like form; M, mycelial form.
[b] 3–4+, bright fluorescence; 2+, moderate fluorescence, 0, no staining.

bedding medium, and sectioned at 4 μm on a Leitz 1512 microtome. Sections were floated on a 40°C water bath, mounted on aminosilane-coated glass microscope slides, warmed in a 65°C paraffin oven for 20 min, and stained with Gomori-methenamine silver. Unstained sections for fluorescent-antibody studies were heated and deparaffinized just before staining.

Immunofluorescence staining. The IFA test was performed with heat-fixed smears prepared with 10 μl of a suspension of fission arthroconidia adjusted to a McFarland no. 1 nephelometer standard. Smears from mycelial cultures were made directly from Sabouraud dextrose agar slant cultures and were heat fixed. Twenty-five microliters of rabbit *P. marneffei* antiglobulin diluted 1:75 in PBS containing 1% bovine serum albumin (PBS-BSA; pH 8.0) was applied to the smears, and the smears were incubated for 45 min at 35°C in a moist chamber. The smears were rinsed with PBS for 10 min and with distilled water for 5 min, after which 25 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit globulin (Centers for Disease Control and Prevention) diluted 1:100 in PBS-BSA at pH 8.0 was added. The same procedure was used with deparaffinized tissue sections after the tissue was treated with 1% trypsin for 45 min (3). The direct staining procedure with specific conjugated antiglobulins for *H. capsulatum* was performed by the method of Kaufman and Kaplan (2). Smears treated with labeled antiglobulins were examined with a Leitz Ortholux II incident-light fluorescence microscope. The intensity of staining was arbitrarily graded. Results were recorded as 0 for negative, 1+ to 2+ for moderate staining, and 3+ to 4+ for strong staining.

Adsorption procedure. Yeast-form antigens of *H. capsulatum* (strain *thon*) for adsorption of antiglobulins were prepared by cultivation for 7 days in PB at 37°C. The culture was killed with Merthiolate (0.02%), incubated for an additional 48 h at 37°C, and then checked for sterility. Pure and sterile cells were harvested and washed three times in PBS. Three volumes of *P. marneffei* antiglobulin were adsorbed with one volume of packed *H. capsulatum* yeast-form cells for 2 h at 37°C.

RESULTS

Rabbit antiglobulins produced against *P. marneffei* ATCC 18224 culture filtrate and whole-cell antigens were evaluated in the IFA procedure for their ability to stain *P. marneffei* fission arthroconidia and mycelial forms and elements of a variety of other fungi. Antibodies to both antigen preparations stained the yeast forms of *H. capsulatum* and the fission forms of *P. marneffei* but not their mycelial forms (Table 1). Furthermore, no staining reactions were observed with the other heterologous yeast- and mycelial-form fungi studied. Included among the latter were two strains each of *Aspergillus flavus* and *Aspergillus fumigatus* and five monomorphic *Penicillium* spp. obtained from Thailand, which grossly and microscopically resembled the mycelial form of *P. marneffei*. A single adsorption of the *P. marneffei* whole-cell or filtrate antibodies with yeast-form cells of *H. capsulatum* completely eliminated the cross-staining of the various *H. capsulatum* strains. Preliminary titrations with the adsorbed antiglobulins in the IFA test indicated that a 1:75 dilution was optimal for both reagents for staining smears of *P. marneffei* fission arthroconidia and cellular elements in tissue.

TABLE 2. Fluorescent-antibody staining reactions of fission arthroconidia of *P. marneffei* and *H. capsulatum* with adsorbed tissue-form *P. marneffei* antiglobulins and an *H. capsulatum* conjugate

Fungus	No. of yeast isolates studied	Fluorescence to[a]:		
		Filtrate antiglobulins	Whole-cell antiglobulins	*H. capsulatum* conjugate
P. marneffei	43	2–4+	1–2+	0
H. capsulatum	5	0	0	2–3+

[a] 3–4+, strong fluorescence; 1–2+, moderate fluorescence; 0, no staining.

An extensive evaluation of these adsorbed *P. marneffei* antiglobulins diluted 1:75 for use in the IFA test in parallel with the specific *H. capsulatum* fluorescent-antibody reagent (2) was performed with 43 isolates of *P. marneffei* received from Thailand and converted to the tissue form in our laboratory and with 5 yeast-form isolates of *H. capsulatum* (Table 2). All 43 *P. marneffei* human isolates stained positive with the adsorbed *P. marneffei* antiglobulins and negative with the *H. capsulatum* conjugate. Moderate homologous staining was retained with the adsorbed antiglobulins produced against *P. marneffei* whole-cell antigens, whereas strong homologous staining was retained with the adsorbed antiglobulins to the *P. marneffei* filtrate antigens. The five *H. capsulatum* isolates were not stained with the adsorbed *P. marneffei* antiglobulins but stained positive with the *H. capsulatum* conjugate (Table 2).

Additional fluorescent-antibody studies were performed to determine the ability of the adsorbed antiglobulins to the filtrate antigens and the specific *H. capsulatum* fluorescent-antibody reagent to react with homologous and heterologous fungi in tissue. Fluorescent-antibody studies were performed with 10 mock or synthetic tissue sections (7) containing yeast-like elements of 10 different isolates of *P. marneffei* as well as with tissue sections obtained from humans with penicilliosis marneffei and histoplasmosis (Table 3). All of the tissues used were stained with Gomori methenamine-silver to verify that each contained yeast-like fungi. Tissue sections from six different patients with disseminated penicilliosis marneffei were studied. Three sections represented skin and subcutaneous tissue, two sections represented lung tissue, and one section was from a cervical lymph node. The *P. marneffei* elements in the 10 simulated tissue sections as well as those from six patients with disseminated penicilliosis marneffei stained positive with the adsorbed antiglobulin and negative with the *H. capsulatum* conjugate. None of the tissue sections from 10 humans with histoplasmosis stained with the adsorbed *P. marneffei* an-

TABLE 3. Fluorescent-antibody staining reactions of the tissue-form *P. marneffei*-specific antiglobulin and *H. capsulatum* conjugate with *P. marneffei* and *H. capsulatum* in tissues

Fungus in tissue sections	No. of isolates or samples tested	Fluorescence to[a]:	
		P. marneffei filtrate antiglobulin	*H. capsulatum* conjugate
P. marneffei in simulated tissue	10	3–4+	0
P. marneffei in human tissue	6	3–4+	0
H. capsulatum in human tissue	10	0	3–4+

[a] 3–4+, positive bright fluorescence; 0, no staining.

tiglobulin, whereas all stained positive with the *H. capsulatum* conjugate (Table 3).

DISCUSSION

Our IFA studies with antiglobulins prepared against whole cells and culture filtrates of the tissue form of *P. marneffei* indicated that both antigen preparations share epitopes with the yeast form of *H. capsulatum*. Interestingly, the tissue form of *P. marneffei* does not appear to share antigens with morphologically similar yeasts, such as *Candida albicans*, *Candida glabrata*, or *Cryptococcus neoformans*. Furthermore, the lack of staining by antiglobulins to the tissue form suggests that the tissue form of *P. marneffei* does not share antigens with its mycelial anamorph (Table 1).

In addition, *P. marneffei* does not appear to share antigens with other species of *Penicillium*. Our findings are consistent with those of Sekhon et al. (6), who reported no reactivity when rabbit antisera to mycelial elements of *P. marneffei* were tested with the exoantigens of eight monomorphic species of *Penicillium.*

Recent studies by Pierard et al. (4) indicated that a monoclonal antibody to *A. fumigatus* galactomannan specifically reacted with *Aspergillus* spp. and *P. marneffei* in formalin-fixed, paraffin-embedded tissues. Similarly, Van Cutsem et al. (8) detected a common circulating antigen in experimentally infected guinea pigs with penicilliosis marneffei and aspergillosis using a latex reagent for *Aspergillus* galactomannan. Contrary to these findings, our antiglobulins produced against tissue-form culture filtrate and cellular antigens of *P. marneffei* (Table 1) as well as those produced against mycelial culture antigens (6) demonstrated no cross-reactivity with hyphal antigens of *Aspergillus* spp. It appears that the common antigen shared by *Aspergillus* spp. and *P. marneffei* is not highly antigenic in *P. marneffei*, since rabbit antiglobulins to the fission arthroconidia and culture filtrate antigens, as well as to mycelial culture filtrate antigens, do not contain antibodies reactive with *Aspergillus* antigens.

Our results revealed that specific antiglobulins useful in IFA tests for rapidly identifying the tissue form of *P. marneffei* could be produced by adsorption with yeast-form cells of *H. capsulatum*. Adsorbed antiglobulins produced against the yeast-like culture filtrate antigens of *P. marneffei* proved to be sensitive and accurate in the IFA test for detecting the pathogen in smears of 43 yeast-like isolates and in tissue sections from six humans with penicilliosis marneffei and 10 mock tissue preparations previously shown to be positive with the Gomori-methenamine silver stain (Tables 2 and 3). No false-positive staining occurred with smears or tissue sections containing yeast-form cells of *H. capsulatum.*

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