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Immunoreactivity of a 38-Kilodalton *Penicillium marneffei* Antigen with Human Immunodeficiency Virus-Positive Sera

P. CHONGTRAKOOL,[1] S. C. CHAIYAROJ,[1] V. VITHAYASAI,[2] S. TRAWATCHAREGON,[1] R. TEANPAISAN,[3] S. KALNAWAKUL,[3] AND S. SIRISINHA[1,4]*

Department of Microbiology, Faculty of Science, Mahidol University,[1] and Laboratory of Immunology, Chulabhorn Research Institute,[4] Bangkok, Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai,[2] and Prince of Songkla University, Songkla,[3] Thailand

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Penicillium marneffei **produced and secreted a 38-kDa antigen that appeared to be specific for this dimorphic fungus. This component could not be detected in antigenic extracts of *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Candida albicans*, and two other species of *Penicillium* by immunoblot analysis against the sera from patients with culture-confirmed penicilliosis marneffei. Antibody reactive with this antigen was found in a large proportion of human immunodeficiency virus (HIV)-positive patients, indicating a presumptive diagnosis of *P. marneffei* infection. A small number of asymptomatic HIV-seropositive patients and HIV-seropositive patients with other fungal infections were also found to be positive by this analysis, suggesting that subclinical or mixed fungal infections involving *P. marneffei* are not uncommon.**

Penicillium marneffei is a dimorphic fungus that was once considered a rare human pathogen, predominantly affecting the residents of southeast Asia and southern China (6). The disease, penicilliosis marneffei, has been reported in both healthy and immunocompromised hosts (6). It is the third most prevalent opportunistic infection in human immunodeficiency virus (HIV)-infected patients from the northern part of Thailand (4, 13). In fact, the Thai Ministry of Public Health now considers it to be an AIDS-defining opportunistic infection (14). The organisms can most often be isolated from blood, lymph nodes, spleen, lung, and bone marrow (6, 13). They have also been recovered from the internal organs of healthy bamboo rats captured in the area where the infection is endemic (1, 5). Histopathological identification is difficult, particularly distinguishing it from *Histoplasma capsulatum*. Immunological identification of the fungus can be done by an immunofluorescence test with polyclonal rabbit antiserum produced against the yeast form from a culture filtrate (8). Several groups of investigators have reported the use of serological methods for the detection of antibody and antigen in patients with culture-confirmed penicilliosis marneffei (8, 9, 15, 17).

P. marneffei is the only known dimorphic *Penicillium* species. It is immunologically distinct from all other *Penicillium* species (3, 8). Currently, very little is known about the composition and characteristics of *P. marneffei* antigens. Different lines of evidence indicated that the mycelial and the yeast forms of *P. marneffei* possessed distinct sets of antigens (8, 9). Using rat monoclonal antibody to galactomannan from *Aspergillus fumigatus*, Latge and associates (11) found the galactofuranosyl side chains of *Penicillium charlesii* to be immunologically related to those of *A. fumigatus*. In the present study, we reported the presence of a *P. marneffei* component that appeared to be highly immunogenic in humans and evaluated its potential for the detection of penicilliosis marneffei.

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand. Fax: 662-644 5411 (662-247 1222). E-mail: SCSSR@MAHIDOL.AC.TH.

MATERIALS AND METHODS

Fungal strains. Four standard strains of *P. marneffei* (ATCC 64102, ATCC 18224, ATCC 24100, and ATCC 64101) and 11 local strains (9 isolates from humans with naturally acquired infections from different parts of the country and 2 isolates from the lungs of two bamboo rats) were used in this study. All strains were morphologically and biochemically indistinguishable from one another. Other fungi including *Aspergillus niger*, *A. fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Histoplasma capsulatum*, *Candida albicans*, *Cryptococcus neoformans*, *Penicillium chrysogenum*, and one other *Penicillium* sp. were from stock cultures kept at the Department of Pathology, Faculty of Medicine, Ramathibodi Hospital (Mahidol University). All mycelial fungi were cultured and maintained under aerobic conditions on Sabouraud dextrose agar (SDA) at 25°C, while the monomorphic yeasts were grown under the same conditions but at 37°C.

Antigen preparation. All mycelial fungi were cultured on SDA and incubated at 25°C for 5 to 7 days. However, for *H. capsulatum*, the incubation period was extended to 21 days due to its slow growth. Monomorphic yeasts and the *P. marneffei* yeast form were cultured at 37°C for 3 days on SDA supplemented with 10% fetal calf serum and 0.1% cysteine and on brain heart infusion agar supplemented with 2% glucose. The growing cells were then subcultured in Sabouraud dextrose broth for mycelial fungi and monomorphic yeasts and in yeast-peptone broth for the *P. marneffei* yeast form. All liquid cultures were placed on a gyratory shaker set at 150 rpm for 6 weeks and were incubated at room temperature for mycelial fungi or at 37°C for yeasts. At the end of the incubation period, Merthiolate at a final concentration of 0.01% was added to each broth culture, and incubation was continued for another 24 h. The cultures were then filtered with 0.45-μm-pore-size Whatman filters. The filtrates were precipitated by the addition of cold acetone (1:3; vol/vol), followed by standing overnight at 4°C. The solutions were then centrifuged at 2,000 rpm at 4°C for 1 h. The pellets were air dried at room temperature for a few hours to allow the residual acetone to evaporate before phosphate-buffered saline (pH 7.2) was added (1:20 of the original volume). The 20× concentrated culture filtrates were then dialyzed against a large volume of phosphate-buffered saline at 4°C, sterilized by filtration through a 0.45-μm-pore-size membrane, and kept in small aliquots at −20°C.

In addition to the culture filtrate antigens, exoantigen and crude whole-cell antigens were used in some experiments. The protocols for the preparation of these antigens were essentially the same as those described by Kaufman and Standard (7).

Serum specimens. A total of 579 HIV-positive serum specimens were available for analysis. Of these, 65 were from HIV-seropositive adult Thai patients with culture-confirmed penicilliosis marneffei, a majority of whom were from the area where the disease is endemic (see Table 1). Four hundred thirty specimens were from asymptomatic HIV-seropositive individuals (262 from individuals from areas where the disease is endemic and 168 from individuals from areas where the disease is not endemic). An additional 84 specimens were from HIV-seropositive patients with other fungal infections including cryptococcosis, candidiasis, and histoplasmosis. The single serum specimen used as a positive control throughout this study was from an HIV-seronegative patient with culture-confirmed penicilliosis marneffei. Negative controls consisted of serum specimens

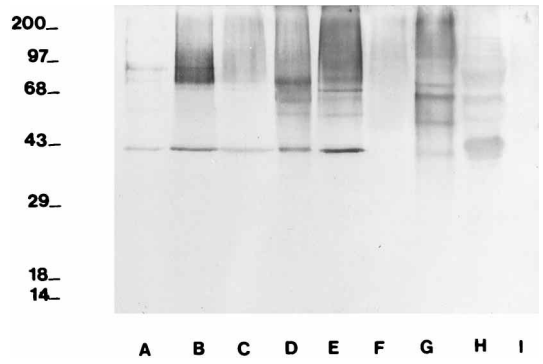

FIG. 1. Identification of the 38-kDa antigen by SDS-PAGE and immunoblotting. Concentrated culture filtrates from the yeast form (lanes A to C) and the mycelial form (lanes D and E) of *P. marneffei* and the mycelial forms of *H. capsulatum* (lanes F and G), *A. niger* (lane H), and one other species of *Penicillium* (lane I) were subjected to SDS-PAGE and then blotted onto a nitrocellulose membrane. The presence of the 38-kDa antigen was detected by probing with human serum from a patient culture positive for *P. marneffei* (diluted 1:1,000). The 38-kDa component was present only in *P. marneffei* culture filtrates. The numbers on the left are molecular sizes (in kilodaltons).

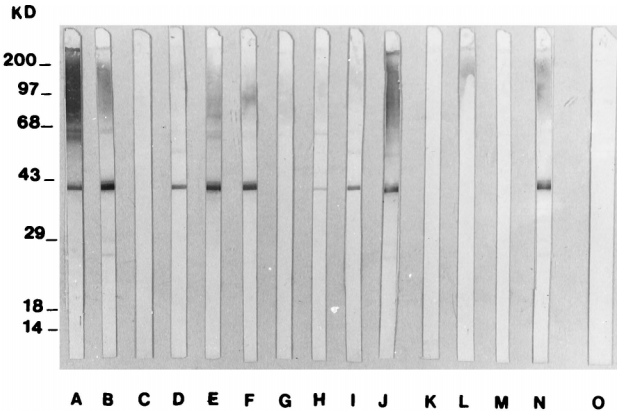

FIG. 2. Representative immunoblot profiles of sera from HIV-seropositive patients with culture-confirmed penicilliosis marneffei. The antigen used was *P. marneffei* culture filtrate. After electrophoresis and blotting, the membrane was allowed to react with reference serum from an HIV-negative patient with penicilliosis (lane A), serum from an HIV-positive patient with penicilliosis (lanes B to N), and serum from a healthy individual (lane O); all sera were diluted 1:1,000, as the legend to Fig. 1. Antibody to the 38-kDa antigen was present in lanes A, B, D, E, F, H, I, J, and N.

from healthy adults and blood donors from areas where *P. marneffei* infection is endemic (60 samples) and not endemic (183 samples).

Characterization of *P. marneffei* antigens. The various forms of *P. marneffei* extracts and secreted products were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of a reducing agent and sodium dodecyl sulfate (SDS), as originally described previously (10). After electrophoresis, the gel was stained with Coomassie blue for the detection of protein or was immediately transferred to a 0.45μm-pore-size nitrocellulose membrane. The membrane was then stained with concanavalin A for the detection of glycoprotein or was probed with human anti-*P. marneffei* serum (diluted 1:1,000). The latter was then subjected to an immunoenzymatic reaction with horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Dako A/S, Copenhagen, Denmark). The molecular masses of these fungal components were determined as described by Weber and Osborn (16).

Immunoreactivity of the 38-kDa antigen with HIV-seropositive sera. The interaction of *P. marneffei* antigens with sera from various groups of HIV-infected patients was detected by the immunoblot analysis described earlier. Although the amount of fungal antigen used was 30 μg/lane in initial experiments, as little as 5 to 10 μg/lane in subsequent experiments gave similar results. To obtain a satisfactory immunoblot pattern, both the positive reference serum and HIV-positive serum could be diluted up to 1:10,000 or 1:20,000. However, in a typical run, the serum was diluted 1:1,000.

Other techniques. The protein and carbohydrate contents of the fungal antigenic preparations were determined by a Folin-Ciocalteau tyrosine method (12) and an orcinol-sulfuric acid method (17), respectively.

RESULTS

Characterization of the 38-kDa immunoreactive component. The immunoblot profiles of crude culture filtrates of the mycelial and yeast forms of *P. marneffei* with the positive reference serum are presented in Fig. 1. Although a number of high-molecular-mass immunoreactive bands could be noticed, the one migrating to a position of 38 kDa was the sharpest and strongest. This prominent immunoreactive component was produced in large quantities in the culture media of both the mycelial and the yeast forms but could also be detected in smaller quantities in cell homogenates (data not presented). Thus, an acetone-precipitated culture filtrate of the mycelial form was used thereafter in the remaining work described in this report. All 11 local strains and reference strains from the American Type Culture Collection produced and secreted this component in various amounts, as judged from the intensity of the immunoreactive band. A faint concanavalin A-positive staining was also detected at this position. Attempts to determine the cellular location of this component by an indirect immunofluorescence test with positive reference serum suggested that it was produced largely by germinating conidia (unpublished data). The mycelial forms of two other species of *Penicillium* (Fig. 1, lane I) and other fungi tested, including *Histoplasma*, *Cryptococcus*, and *Candida* spp., did not produce this 38-kDa component that could be detected by immunoblot analysis. However, very faint, ill-defined bands (one above and one below the 38-kDa position) were occasionally detected at this position in the culture filtrates of *A. niger* (Fig. 1, lane H), but not in those of three other *Aspergillus* species tested (data not shown).

Detection of antibody to the 38-kDa antigen in HIV-positive sera. Representative immunoblot profiles of the sera from various groups of HIV-seropositive patients are presented in Fig. 2 and 3. The results summarized in Table 1 indicate that a majority of serum specimens from the group with culture-confirmed penicilliosis reacted strongly with the 38-kDa component, regardless of their origin. In contrast, a much smaller proportion of specimens from the asymptomatic group was found to be positive by this method. In such cases, the intensity of the band was also rather weak (Fig. 3). It was of consider-

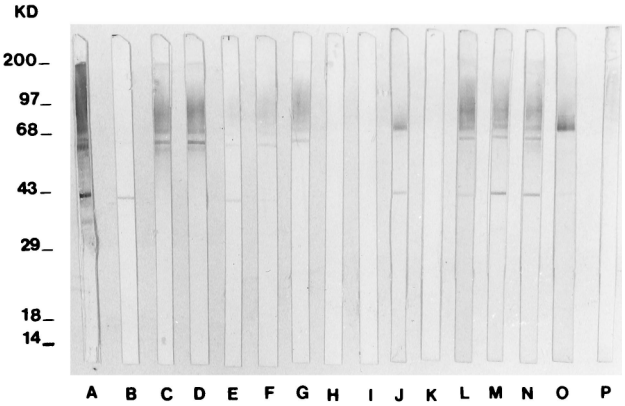

FIG. 3. Representative immunoblot profiles of sera from asymptomatic HIV-positive patients living in an area where the infection is endemic (lanes B to O). Lanes A and P, positive and negative controls, respectively. See legends to Fig. 1 and 2 for other explanations. A faint positive band at the 38-kDa position was noticed in lanes B, E, J, M, and N, in contrast to the very strong reaction in lane A and in sera from a HIV-positive patient with penicilliosis marneffei in Fig. 2.

TABLE 1. Presence of antibody to the 38-kDa *P. marneffei* antigen in sera from HIV-seropositive patients and seronegative controls

Patient status	No. of patients from the following areas positive for anti-38-kDa antigen/no. tested (%)	
	Endemic for *P. marneffei*	Not endemic for *P. marneffei*
HIV positive and culture-confirmed for the following:		
Penicilliosis	23/51 (45)	7/14 50)
Cryptococcosis	11/39 (28)	0/14 (0)
Candidiasis	6/28 (21)	1/1
Histoplasmosis	—[a]	0/2
Total	40/118 (34)	8/31 (26)
Asymptomatic HIV positive	45/262 (17)	1/168 (<1)[b]
Healthy	1/60 (<2)	0/183 (0)

[a] —, no samples were available for testing.
[b] Six samples had questionable results because a clear band at the 38-kDa position could not be identified.

able interest that except for one sample, these anti-38-kDa-positive sera were from asymptomatic HIV-seropositive patients residing in the area where *P. marneffei* is endemic. A few serum specimens from the HIV-seropositive patients with other mycoses were also antibody positive for the 38-kDa antigen (Table 1). Taken together, the proportion of sera from symptomatic HIV-seropositive patients reacting with this antigen was three times higher than that from asymptomatic HIV-seropositive patients (32 versus 11%). Unlike the HIV-positive sera, all but one serum specimen from a healthy individual gave a negative reaction for antigen at this position.

DISCUSSION

The results presented in this report indicate that a 38-kDa antigen is specifically produced by *P. marneffei* and is highly immunogenic for humans. Different lines of evidence presented suggested that it is most likely associated with the cell envelope, but it may also have been secreted in soluble form by the fungi grown as either the mycelial or the yeast form. Both the cell-associated and the secreted soluble products appeared to have the same molecular size. It was initially thought that this component represented a galactomannan-like substance, like that occasionally detected in *Aspergillus* cultures. For example, Latge and associates (11) previously reported that rat monoclonal antibody to *Aspergillus* galactomannan cross-reacted immunologically with extracts of *Penicillium* species. In their test system, a majority of the immunoreactive components had molecular weights larger than 35,000. In fact, the majority of the cross-reactive components in that report had relatively high molecular masses, i.e., in the range of 100,000 Da. Similarly, another monoclonal antibody against *Aspergillus* galactomannan could detect *P. marneffei* in a biopsy specimen from an experimentally infected guinea pig (2). With our system, however, no cross-reaction was noted with any of the four *Aspergillus* species used. The smearing reaction noted with *A. niger* (Fig. 1, lane H) was different from the reaction resulting in the 38-kDa component reported in this study. A faint band at a position slightly below the 38-kDa position, noted with the *H. capsulatum* filtrate (Fig. 1, lane G), was most likely different from the *P. marneffei* antigen because the serum from a histoplasmosis patient did not react with *P. marneffei* at this position (unpublished data).

Although the exact nature of this 38-kDa antigen remains to be elucidated, it was nevertheless highly immunogenic in humans (Table 1). BALB/c mice experimentally infected or immunized with the fungus also produced a strong antibody response to the antigen at this position (unpublished data). The fact that for only one-half of the HIV-seropositive patients with confirmed penicilliosis marneffei was antibody detectable in their sera may be related to different degrees of immunological defect in these patients. The presence of antibody in small group of asymptomatic HIV-seropositive patients may be related to subclinical infection, because almost all of them were from the area where penicilliosis marneffei is endemic. It should be very interesting to follow these *P. marneffei*-positive asymptomatic patients and to see if they would subsequently develop clinical penicilliosis marneffei. The data summarized in Table 1 also suggested that simultaneous mixed fungal infections in HIV-seropositive patients are not uncommon in this part of the world.

The presence of antibody to this *P. marneffei* antigen in a large proportion of HIV-seropositive patients can be used as a presumptive diagnosis for previous or current infection with *P. marneffei*. In patients with acute infections, proper antifungal therapy could be initiated while waiting for the results of culture and species identification. In those without clinical penicilliosis marneffei, physicians can closely monitor the patients and proper treatment can be given as soon as possible when clinical disease develops. In a limited number of patients, other investigators also reported the detection of antibody to *P. marneffei* in HIV-seropositive patients by immunodiffusion and immunofluorescence techniques (8, 9, 15, 18). On the other hand, Kaufman and associates (9) found that only a small proportion of HIV-seropositive patients had antibody to *P. marneffei*, but instead, they reported satisfactory results for the detection of antigenemia by the latex agglutination test with sera from these patients. If the results could be confirmed with a larger group of patients, it might be of high diagnostic valuable. In our limited trials by the technique, we failed to detect *P. marneffei* antigen in our group of patients. However, progress is being made to improve the sensitivity of methods for antigen detection.

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