

PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST A YEAST SECRETED ANTIGEN OF *PENICILLIUM MARNEFFEI*

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Abstract. Monoclonal antibody against *P. marneffei* yeast secreted antigen was produced in order to develop a serological test for penicilliosis marneffei. The yeast form of *P. marneffei* was cultured in brain heart infusion broth at 37°C for 7 days. A secreted antigen was prepared, partially purified from culture supernatant and subsequently immunized in a BALB/c mouse. Mouse monoclonal antibody was produced from immune spleen cells by a standard hybridoma technique. Specificity of the obtained monoclonal antibody was assessed with yeast secreted antigens for *P. marneffei*, *C. albicans*, *C. neoformans*, and *H. capsulatum* by an indirect ELISA. Three of 46 hybrid clones (1F1, 2G5, and 3G4) reacted positively with *P. marneffei* secreted antigen. 1F1 and 3G4 were cloned by two rounds of limiting dilution. Partially purified monoclonal antibody and rabbit polyclonal antibody against *P. marneffei* yeast secreted antigen were used to develop a double antibody sandwich ELISA to detect *P. marneffei* antigen in plasma or serum samples of 7 patients with penicilliosis marneffei and 5 healthy controls. The sandwich ELISA developed using monoclonal antibody as a capture antibody and rabbit polyclonal antibody as a detector was able to detect *P. marneffei* antigen in all the plasma and serum samples of penicilliosis marneffei patients, while negative in all the healthy controls. Thus, the monoclonal antibody produced in the present study appeared to be highly specific for *P. marneffei* and the double antibody sandwich ELISA developed using monoclonal and polyclonal antibodies against the yeast secreted antigen of *P. marneffei* showed a strong potential for the diagnosis of penicilliosis marneffei.

INTRODUCTION

Penicillium marneffei is a thermally dimorphic fungus in the genus *Penicillium* that has received considerable attention in the last few years as it is a causative agent of an important emerging opportunistic fungal infection in people with human immunodeficiency virus (HIV) infection, especially HIV infected patients living in Southeast Asia and South China (Deng *et al*, 1988; Li *et al*, 1992; Supparatpinyo *et al*, 1992, 1994; Borrado *et al*, 1994). The diagnosis of penicilliosis marneffei is difficult because its clinical manifestations mimic those of tuberculosis, pneumocystosis, histoplasmosis and several other mycotic infections, all of these are also observed in HIV-infected patients. Although the staining of a specimen is a rapid diagnostic

method, the tissue form of *P. marneffei* can be confused with those of *Histoplasma capsulatum* and *Cryptococcus neoformans* (Trewatcharegon *et al*, 2000). The culture of *P. marneffei* from a specimen is the standard diagnostic method. However, the major disadvantage of this method is that it requires a long culture time and a delay in the initiation of appropriate treatment. In addition, the disease is associated with a high mortality rate (Imwidthaya, 1994; Duong, 1996). A number of presumptive diagnostic methods have been developed, including serology, but a problem is a cross reaction between the antigen of *P. marneffei* and other fungi, such as *A. fumigatus*, *C. albicans*, and *C. neoformans* (Arrese Estrada *et al*, 1992; Yuen *et al*, 1994; Vanittanakom *et al*, 1997). Monoclonal antibody reaction with the mycelial culture filtrate of *P. marneffei* has been produced and used to detect the antigen in a patient's serum (Trewatcharegon *et al*, 2000; Chaiyaroj *et al*, 2003). Since *P. marneffei* grows as a yeast in the human body and the monoclonal antibody reaction to yeast secreted antigen has not been stud-

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ied yet, we aimed to develop a monoclonal antibody against the yeast secreted antigen for the detection of *P. marneffei* antigenemia in the plasma and serum samples of penicilliosis marneffei patients by the sandwich ELISA technique.

MATERIALS AND METHODS

Preparation of secreted fungal antigens

Yeast cells of *P. marneffei* (strain 673H) and *C. albicans*, *C. neoformans*, and *H. capsulatum* growing on the surface of brain heart infusion (BHI) agar slant were suspended in 5 ml of phosphate buffer saline solution (PBS) and transferred to 50 ml BHI broth. After 7 days of incubation at 37°C, the culture supernatants were collected by centrifugation at 20°C 150g for 15 minutes. The protein antigens in the culture supernatant were saturated by 70% ammonium sulfate and dialyzed overnight in 0.1% NaCl. The concentration of protein antigens was detected by Lowry's method (Lowry *et al*, 1951) and kept in small aliquots at -20°C.

Production of monoclonal antibodies

Five-week old female BALB/c mice were immunized with 28 µg/100 µl of 70% ammonium sulfate saturated culture supernatant protein of *P. marneffei* strain 673H. The protein antigen was immunized intraperitoneally for 6 weeks followed by intravenously for 3 days before fusion. Antibodies specific for *P. marneffei* in mouse serum and hybrid culture supernatant were screened by indirect ELISA. Serum from immunized mice and the hybridoma culture fluids were added to the protein antigen coated plate and the reaction was detected with horseradish peroxidase conjugated rabbit-anti-mouse immunoglobulin. The clone secreting antibodies which cross reacted with other fungal antigens were screened out by indirect ELISA using a panel of fungal antigens prepared from culture supernatants of the yeast forms of *C. albicans*, *C. neoformans*, and *H. capsulatum*. Clones that reacted specifically to *P. marneffei* antigens were propagated. Two clones (1F1 and 3G4) which reacted strongly and specifically to *P. marneffei* antigens were selected for limiting dilution. After two rounds of limiting dilution, the 1F1-E9-D5 clone that gave the strongest reaction with *P. marneffei* antigens was selected. The culture supernatant from this clone was collected and precipitated with satu-

rated ammonium sulfate, dialyzed and concentrated by lyophilization. This monoclonal antibody was used for the detection of *P. marneffei* antigenemia in the serum and plasma of penicilliosis marneffei patients by the double antibody sandwich ELISA technique.

Detection of *P. marneffei* antigenemia

By double antibody sandwich ELISA technique, monoclonal antibody prepared from the 1F1-E9-D5 clones was coated onto a microtiter plate. Fifty microliters of patient diluted plasma or serum (plasma or serum: PBS was 1:500) was added to the plate and incubated at room temperature for one hour. Unbound samples were removed by washing and rabbit-anti-*P. marneffei*-antibody was added and incubated at room temperature for another hour. After washing to remove any unbound rabbit-anti-*P. marneffei*-antibody, 100 µl of the conjugate was added, incubated, washed, and followed with 100 µl of substrate solution. After incubation at room temperature for 30 minutes in the dark, the reaction was stopped with 100 µl of 1 N sulfuric acid. The optical density of each well was determined within 30 minutes by microplate reader (Thermo labsystems, Dymex Technologies, MRX Revelation) at 450/570 nm.

RESULTS

There were 3 clones (1F1, 2G5, and 3G4) that reacted strongly with the yeast secreted antigen of *P. marneffei*. However, the 2G5 clone was screened out because of a cross reaction with secreted antigen of *C. albicans* (Fig 1). 1F1 was chosen for limited dilution, and the 1F1-E9-D5 that had the strongest reaction to *P. marneffei* was selected for the detection of *P. marneffei* antigen in the serum and plasma of penicilliosis marneffei patients. The results showed that the culture supernatant from this clone could detect *P. marneffei* antigen in either the serum or the plasma of all the penicilliosis marneffei patients, with a mean OD which was significantly higher than that of the serum of healthy persons ($p < 0.004$ and 0.00017 with patient's serum and plasma, respectively) but there was no significant difference between patient's serum and plasma ($p = 0.23$) (Fig 2).

DISCUSSION

Effective serodiagnosis of systemic fungal

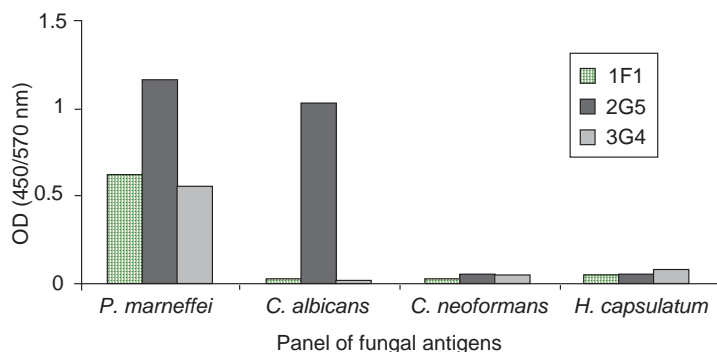


Fig 1—Optical density of indirect ELISA for the screening of 3 clones secreting antibody to *P. marneffeii* against a panel of fungal antigens.

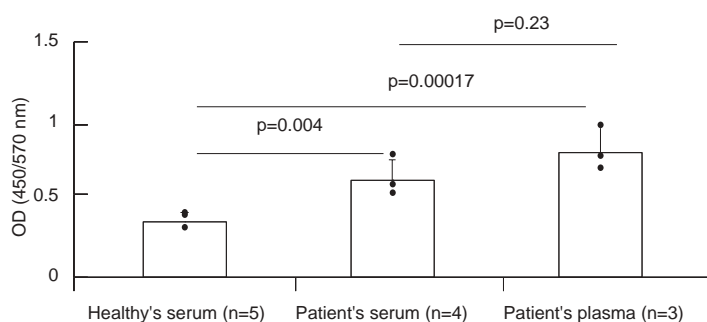


Fig 2—Mean \pm SD optical density of *P. marneffeii* antigenemia assayed in serum and plasma of patients with penicilliosis marneffeii, compared to healthy patient's serum by the double antibody sandwich ELISA technique (p-value derived from Student's *t*-test).

infections is of increasing importance, particularly in regard to the identification of infection with *P. marneffeii*. Diagnosis has been based on either antibody or antigen detection. Western blot and ELISA for the detection of anti-*P. marneffeii* antibodies in a patient's serum or plasma have been developed, but the specificity of the tests has been low and some normal serum and plasma samples have given positive results (Vanittanakom *et al*, 1997; Sariya *et al*, 2000). This supports the hypothesis that healthy people have been exposed to this fungus but have had no symptoms due to immune function (Sariya *et al*, 2000). In addition, other limitations, due to cross-reactivity with other fungal pathogens and the majority of immunosuppressed AIDS patients have given abnormal antibody responses (Hamilton, 1998). In the case of antigen detection, an immunodiffusion assay and a latex agglutination test, which use polyclonal antibodies

against a yeast culture filtrate of *P. marneffeii* have developed. However, the sensitivity of both the tests has been rather low (Van Cutsem *et al*, 1990; Arrese Estrada *et al*, 1992; Kaufman *et al*, 1996). A high sensitivity and specificity test for *P. marneffeii* infection has been developed using monoclonal antibodies. Arrese Estrada and colleagues indicated that the monoclonal antibody EB-A1 to galactomannan of *A. fumigatus* together with an immunohistochemical technique can be useful in the diagnosis of *P. marneffeii* antigen in both patient's tissues and phagocytic cells (Arrese Estrada *et al*, 1992). The recent utilization of monoclonal antibodies in the development of antigen detection methods for the diagnosis of penicilliosis marneffeii offers further hope for improvement in this area. The results indicated that the hybrid clone produced antibodies against yeast secreted antigen for *P. marneffeii* without reacting with the antigens derived from culture supernatant of the yeast forms of *C. albicans*, *C. neoformans*, and *H. capsulatum*. By the double antibody sandwich ELISA, monoclonal antibody can detect *P. marneffeii* antigen in both serum and plasma of penicilliosis marneffeii but not in the serum of healthy individuals. These results correlate with a previous study; monoclonal antibody against mycelial culture filtrate of *P. marneffeii* was highly reactive with mycelial and yeast exoantigens of this fungus but did not react with the antigens of *C. albicans*, *C. neoformans*, *H. capsulatum*, *A. fumigatus*, and *A. flavus*. In addition, this monoclonal antibody could detect *P. marneffeii* antigens in the serum of patients with penicilliosis marneffeii, but not in the serum of patients with candidiasis, cryptococcosis, histoplasmosis, or healthy persons (Trewatcharegon *et al*, 2000; Chaiyaraj *et al*, 2003). In that study, the monoclonal antibody was produced by immunization of BALB/c mice with mycelial culture filtrate of *P. marneffeii*, but in our study, we immunized BALB/c mice with

antibody sandwich ELISA, monoclonal antibody can detect *P. marneffeii* antigen in both serum and plasma of penicilliosis marneffeii but not in the serum of healthy individuals. These results correlate with a previous study; monoclonal antibody against mycelial culture filtrate of *P. marneffeii* was highly reactive with mycelial and yeast exoantigens of this fungus but did not react with the antigens of *C. albicans*, *C. neoformans*, *H. capsulatum*, *A. fumigatus*, and *A. flavus*. In addition, this monoclonal antibody could detect *P. marneffeii* antigens in the serum of patients with penicilliosis marneffeii, but not in the serum of patients with candidiasis, cryptococcosis, histoplasmosis, or healthy persons (Trewatcharegon *et al*, 2000; Chaiyaraj *et al*, 2003). In that study, the monoclonal antibody was produced by immunization of BALB/c mice with mycelial culture filtrate of *P. marneffeii*, but in our study, we immunized BALB/c mice with

yeast culture filtrate. This correlates better with the antigen of *P. marneffe* in patient's serum, because it grows in the yeast form in the human body. In the next experiment we will focus on the characteristics of this monoclonal antibody (isotype of immunoglobulin, MW and the nature of the immunoreactive epitope of this specific antigen). Since Qui and colleagues showed that monoclonal antibody against mannoprotein 1 (MP1) can cross-reacted with the antigen of *A. fumigatus* (Qiu *et al*, 2003), cross-reaction with the antigen of *A. fumigatus* should be studied. To obtain more information on the accuracy and precision of the results we will assay larger numbers of *P. marneffe* infected patients, and compare them to other mycosis-infected patients.

In summary, the double antibody sandwich ELISA developed with monoclonal antibodies against yeast secreted antigen of *P. marneffe*, described here should prove useful for the rapid serodiagnosis of *P. marneffe* infection and may be modifiable into a more convenient diagnosis kit. The test should be applicable for the identification of individuals with nonspecific symptoms of *P. marneffe* infection as well as those who have subclinical forms of the disease.

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REFERENCES

- Arrese Estrada J, Stynen D, Van Cutsem J, Pierard-Franchimont C, Pierard GE. Immunohistochemical identification of *Penicillium marneffe* by monoclonal antibody. *Int J Dermatol* 1992; 31: 410-2.
- Borradori L, Schmit JC, Stetzkowski M, Dussoix P, Saurat JH, Filthuth I. Penicilliosis marneffe infection in AIDS. *J Am Acad Dermatol* 1994; 31: 843-6.
- Chaiyaroj SC, Chawengkirttikul R, Sirisinha S, Watkins P, Srinoulprasert Y. Antigen detection assay for identification of *Penicillium marneffe* infection. *J Clin Microbiol* 2003; 41: 432-4.
- Deng Z, Ribas JL, Gibson DW, Connor DH. Infections caused by *Penicillium marneffe* in China and South-east Asia: review of eighteen published cases and report of four more Chinese cases. *Rev Infect Dis* 1988; 10: 640-52.
- Duong TA. Infection due to *Penicillium marneffe*, an emerging pathogen: review of 155 reported cases. *Clin Infect Dis* 1996; 23: 125-30.
- Hamilton AJ. Serodiagnosis of histoplasmosis, paracoccidioidomycosis and penicilliosis marneffe; current status and future trends. *Med Mycol* 1998; 36: 351-64.
- Imwidthaya P. Systemic fungal infections in Thailand. *J Med Vet Mycol* 1994; 32: 395-9.
- Kaufman L, Standard PG, Jalbert M, Kantipong P, Limpakarnjanarat K, Mastro TD. Diagnostic antigenemia tests for penicilliosis marneffe. *J Clin Microbiol* 1996; 34: 2503-5.
- Li PC, Tsui MC, Ma KF. *Penicillium marneffe*: indicator disease for AIDS in South East Asia. *Aids* 1992; 6: 240-1.
- Lowry OH, Rosebrough NJ, Fatt AL, Randall RJ. Protein measurement with the Folin Phenol reagent. *J Biol Chem* 1951; 193: 265-75.
- Qiu LW, Peng DX, Xu H, Jia J, Che XY, Yuen KY. [Preparation and identification of monoclonal antibodies against *Penicillium marneffe*]. *Di Yi Jun Yi Da Xue Xue Bao* 2003; 23: 38-9.
- Sariya L, Klinhom R, Chaiwong T, Pornprasert S. Detection of antibodies against *Penicillium marneffe* antigens in Chiang Mai healthy population. *Bull Chiang Mai Assoc Med Sci* 2000; 33: 172-81.
- Supparatpinyo K, Chiewchanvit S, Hirunsri P, Uthammachai C, Nelson KE, Sirisanthana T. *Penicillium marneffe* infection in patients infected with human immunodeficiency virus. *Clin Infect Dis* 1992; 14: 871-4.
- Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T. Disseminated *Penicillium marneffe* infection in Southeast Asia. *Lancet* 1994; 344: 110-3.
- Trewatcharegon S, Chaiyaroj SC, Chongtrakool P, Sirisinha S. Production and characterization of monoclonal antibodies reactive with the mycelial and yeast phases of *Penicillium marneffe*. *Med Mycol* 2000; 38: 91-6.
- Van Cutsem J, Meulemans L, Van Gerven F, Stynen D. Detection of circulating galactomannan by Pastorex Aspergillus in experimental invasive aspergillosis. *Mycoses* 1990; 33: 61-9.
- Vanittanakom N, Mekaprateep M, Sittisombut N, *et al*. Western immunoblot analysis of protein antigens of *Penicillium marneffe*. *J Med Vet Mycol* 1997; 35: 123-31.
- Yuen KY, Wong SS, Tsang DN, Chau PY. Serodiagnosis of *Penicillium marneffe* infection. *Lancet* 1994; 344: 444-5.