Asian Pacific Journal of Allergy and Immunology (1996) 14 : 43-47

Production of Specific Monoclonal Antibodies to *Burkholderia pseudomallei* and Their Diagnostic Application

Supinya Pongsunk[1], Pattama Ekpo[1] and Tararaj Dharakul

SUMMARY **Hybridomas secreting monoclonal antibodies (MAbs) specific to *Burkholderia pseudomallei* were produced by immunizing BALB/cJ mice with crude culture filtrate of *B. pseudomallei*. Two monoclonal antibodies were found to be highly specific to *B.pseudomallei* as tested by indirect enzyme-linked immunosorbent assay and immunoblotting against a panel of crude whole cell extracts from *B. pseudomallei*, *B. cepacia*, *Pseudomonas aeruginosa*, *P. putida*, and *Escherichia coli*. One of the specific MAbs, clone SP-M, IgM subclass, could directly agglutinate all 42 *B. pseudomallei* isolates. The study has shown that the agglutinating MAb has potential for rapid identification of *B. pseudomallei* in primary bacterial culture from clinical specimens. The antibody can be used in bacteriology laboratories to reduce time of biochemical methods, which require a few days.**

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*. The disease is still important public health problem in Southeast Asia and Northern Australia, particularly with respect to diagnosis and treatment.[1,2] Early diagnosis is necessary for proper management because death may occur within 24 to 48 hours after the onset of infection.[1] Since the clinical manifestations of melioidosis can mimic a number of other infectious diseases, a specific diagnostic method should be employed.[3]

A definitive diagnosis of melioidosis depends on bacterial isolation and identification. As the method is time-consuming (at least 3-4 days) and the result is often too late to be useful, a number of serological tests have been developed to provide rapid and presumptive evidence of the infection.[1,4-14] However, the tests lack diagnostic specificity in endemic areas because of cross-reactivity with other bacteria and high prevalence of antibody in normal populations. The serological tests for melioidosis are only supplementary to bacteriological culture and clinical awareness.

To reduce timing of the identification step and to make the test applicable in field studies, species-specific monoclonal antibodies (MAbs) that can directly agglutinate *B. pseudomallei* should be produced. In the present study, species-specific agglutinating MAbs were established and shown to be useful for rapid identification of the bacteria by direct agglutination.

MATERIALS AND METHODS

Bacterial strains

Forty-two *Burkholderia pseudomallei* strains and 1 strain of *Xanthomonas maltophilia* used in this study were kindly provided by Dr Sirisinha (Faculty of Science, Mahidol University, Bangkok, Thailand). Other bacteria used (1 strain of *Acinetobacter anitratus*, 1 strain of *B. cepacia*, 1 strain of *Enterobacter cloacae*, 1 strain of *Escherichia coli*, 1 strain of *Klebsiella pneumoniae*, 1 strain of *Proteus* spp., 1 strain of *Pseudomonas aeruginosa*, 1 strain

From the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
[1] Present address: Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Prasarnmit, Bangkok 10110, Thailand.
Correspondence : Supinya Pongsunk

of *P. putida,* 5 strains of *Salmonella typhi,* 2 strains of *S. paratyphi* A, 1 strain of *S. paratyphi* B, 1 strain of *S. krefeld,* 1 strain of *S. enteritidis,* 1 strain of *Staphylococcus aureus,* 1 strain of *Streptococcus* group B and 1 strain of *Streptococcus* group D) were from stock cultures maintained at the Department of Microbiology and Department of Immunology of the Faculty of Medicine Siriraj Hospital (Mahidol University, Bangkok, Thailand).

Preparation of antigens

Crude culture filtrate antigen of *B. pseudomallei* was prepared by culturing *B. pseudomallei* in synthetic broth (glycine 1 g, disodium phosphate 0.25 g, sodium chloride 0.5 g and dextrose 0.2 g made to 100 ml with distilled water) at 37°C for 2 weeks. The supernatant was collected by centrifugation at 10000 × g for 30 minutes at 4°C, then filtered through 0.45 μm membrane filter. The preparation was lyophilized and stored at 4°C. This crude culture filtrate antigen was used as an immunogen for the production of MAbs. The whole cell (WC) antigens of *B. pseudomallei, B. cepacia, P. aeruginosa, P. putida,* and *E. coli* used in this study were prepared by growing each of the bacteria in brain heart infusion broth. The bacteria were collected by centrifugation and resuspended in normal saline solution (NSS), then heated for 5 minutes in a boiling water bath. These WC antigens were used in the experiment for screening and specificity test of the MAbs. The amount of the antigen were determined for the protein concentration by protein assay kit (Bio-Rad, USA).

Production of monoclonal antibodies

The crude culture filtrate of *B. pseudomallei* 10 μg protein homoginized in complete Freund's adjuvant were injected intraperitoneally into BALB/cJ mice. Booster immunization was followed 2 weeks later with intravenous injection of 10 μg of the antigen in NSS. Two days later the spleen was removed and fused with myeloma cell line (P3X 63 Ag 8.653). The fusion procedure was carried out as described by Kearney.[15] Hybridomas producing antibodies were screened by indirect ELISA using WC antigen of *B. pseudomallei* and cloned by limiting dilution. MAbs produced from each clone were tested for their specificity by indirect ELISA using a panel of WC. The hybridomas producing MAbs that reacted specifically to only *B. pseudomallei* were cloned again three times and then expanded in 250 ml tissue culture flasks for bulk production. MAbs were partially purified from the culture supernatants by precipitation with 50% ammonium sulfate and the protein concentration was determined.

Screening for specific monoclonal antibodies

Indirect ELISA employing WC antigen of *B. pseudomallei* coated plate was used for screening test and each of a panel WC antigens from *B. pseudomallei, B. cepacia, P. aeruginosa, P. putida,* and *E. coli* was used for specificity test. Twenty micrograms per millilitre of WC antigen were diluted in 0.05 M carbonate buffer (pH 9.6) at 37°C for 3 hours. After washing the plate with 0.05% v/v Tween 20 in normal saline solution (Tween saline) the culture supernatant was added and incubated at 4°C overnight. Alkaline phosphatase conjugated rabbit antimouse immunoglobulin (Dakopatts, Denmark), diluted 1:250 in 1% bovine serum albumin (BSA) in Tween saline was then added and incubated for 1 hour at 37°C. After excess conjugate was removed, the substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma, USA) was added. The enzymatic reaction was allowed to proceed at room temperature for 1 hour. The optical density was measured at 405 nm. A criterion for a positive result was set arbitrarily at 0.2 OD unit above the background that was determined from the mean OD value performed without test supernatant (the mean OD was 0.05).

Characterization of monoclonal antibodies

The isotypes of MAbs were also determined by indirect ELISA as described using rabbit antibodies to mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, κ light chain and λ light chain conjugated to alkaline phosphatase (Bio-Rad, USA). The specificity of the MAbs were further tested by indirect ELISA and immunoblotting against a panel of WC antigens.

SDS-PAGE and immunoblotting

The WC antigen with amounts among 50–100 μg per well was subjects to SDS-PAGE in 10% acrylamide gel and was run at 40 mA constant current per gel slab at 25°C for 3 hours. After electrophoresis, the protein bands were electroblotted onto nitrocellulose membrane in methanol-tris glycine buffer at 6 volts per cm for 16 hours.

The nitrocellulose membrane was rinsed with 1% v/v Tween 20 in PBS (1% PBST) and then immersed in 3% BSA dissolved in 1% PBST (3% BSA-1% PBST) at room temperature for 3 hours. After washing with 1% PBST, the membrane was incubated with the MAb at room temperature for 2 hours, washed again and incubated with 1:1,000 dilution of rabbit antimouse immunoglobulin-alkaline phosphatase conjugate (Dakopatts, Denmark) in 3% BSA-1% PBST at room temperature for 1 hour. The substrate o-dianizidine tetrazotised (6 mg/ml) (Sigma, USA) was added and the reaction was stopped by washing with distilled water.

Direct agglutination test for identification of *B. pseudomallei*

Direct agglutination for identification of *B. pseudomallei* was done by resuspending one colony of the bacteria in 20 μl of NSS on a glass slide. Twenty microliters of selected MAb (5 mg/ml) was added and the bacterial suspension was mixed by rotating the slide for 5 minutes. Direct bacterial agglutination was recorded visually.

Table 1. The reactivity of *B. pseudomallei* specific MAbs against a panel of whole cell antigens by indirect ELISA.

	OD against whole cell antigens of				
	B. pseudomallei	*B. cepacia*	*E. coli*	*P. aeruginosa*	*P. putida*
SP-M	> 2	0.069	0.069	0.075	0.075
SP-A	1.621	0.033	0.036	0.033	0.036

RESULTS

Production and characterization of monoclonal antibodies

MAbs against specific component of *B. pseudomallei* were produced by immunizing the BALB/cJ mice with crude culture filtrate antigen. Culture supernatants from the hybridomas were screened for antibody activity by indirect ELISA against WC antigen of *B. pseudomallei*. Two MAbs secreted from the clones, SP-A and SP-M, which reacted well to *B. pseudomallei* but not to other bacteria were selected. The reactivity of the *B. pseudomallei*-specific MAbs are shown in Table 1. The isotypes of SP-A and SP-M were found to be IgA λ and IgMκ, respectively.

To further explore the specificity and to identify the immunoreactive components of *B. pseudomallei* the crude WC antigens from each of the *B. pseudomallei, B. cepacia, P. aeruginosa, P. putida,* and *E.coli* were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted onto the nitrocellulose membrane, and then probed with the MAbs. The results also showed the reactivity of both MAbs only with *B. pseudomallei*. For the immunoreactive component the result in Figure 1 showed that SP-A and SP-M MAbs reacted with the components that migrated to 41-90 kDa and 42 kDa, respectively.

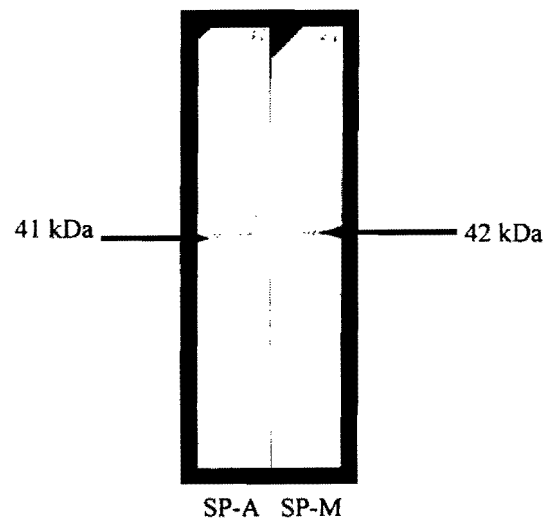

Fig. 1. Immunoblot patterns of *Burkholderia pseudomallei* specific monoclonal antibodies against whole cell antigens.

Direct agglutination for identification of *B. pseudomallei* colony

It was found that MAb SP-M reacted strongly with *B. pseudomallei* in a slide agglutination test, and rapid identification of *B. pseudomallei* colonies on agar plates could be demonstrated. A double-blind experiment was set up and the results obtained from the direct agglutination test were compared with those from the bacteriologic and biochemical identifications as gold standards. The MAb SP-M could agglutinate all 42 *B. pseudomallei* cultures and did not agglutinate any of the 22 bacterial cultures other than *B. pseudomallei* available for testing (Table 2).

To evaluate the stability of the partially purified MAb, MAb SP-M was stored at 4°C and used periodically over an extended time: the MAb could be stored at 4°C for at least 3 months without any loss of reactivity.

Table 2. Identification of *B.pseudomallei* colonies on the agar plates by direct bacterial agglutination using SP-M monoclonal antibody.

Bacteria	No. positive/No. tested
B.pseudomallei	42/42
Acinetobacter anitratus	0/1
B.cepacia	0/1
Enterobacter cloacae	0/1
Escherichia coli	0/1
Klebsiella pneumoniae	0/1
Proteus spp.	0/1
Pseudomonas aeruginosa	0/1
P. putida	0/1
Salmonella spp.	0/10
Staphylococcus aureus	0/1
Streptococcus group B	0/1
Streptococcus group D	0/1
Xanthomonas maltophilia	0/1

DISCUSSION

The monoclonal antibodies obtained in the present study were shown to be highly specific for ***B. pseudomallei*** as determined by indirect ELISA and immunoblotting against a panel of WC antigens prepared from close related bacteria such as ***B. cepacia, P. aeruginosa, P. putida*** and ***E. coli.*** ***B. pseudomallei*** and ***B. cepacia*** are very similar to each other in their biochemical and clinical characteristics, the differential identification of these two bacteria has to be tested by special biochemical tests.[16] The data clearly show that the ***B. pseudomallei*** specific MAb SP-M has potential for use in the rapid identification of ***B. pseudomallei.*** Direct agglutination using this MAb for the identification of ***B. pseudomallei*** can be easily performed.

For further study, the MAb SP-M will be modified by coupling to protein-A bearing ***S. aureus*** or latex particles to improve the sensitivity of the test,[17] to enable specific bacterial antigen to be directly detected in whole blood or in short term hemoculture broth.

The evidence suggested that the immunoreactive components of these specific MAbs were protein, because we previously noted that the specific epitopes of both MAbs were destroyed by proteinase K digestion (unpublished data). Using the combination of the MAbs (SP-A or SP-M) and recombinant DNA technology, the specific protein components of ***B. pseudomallei*** will be cloned and expressed in ***E. coli.*** A genomic DNA library of ***B. pseudomallei*** has been constructed and screened for the expressed recombinant clones using the specific MAbs in our laboratory. These purified antigens may be used for detection of specific antibody in patients' sera. Recently, Rugdech *et al* reported the ***B. pseudomallei*** specific MAb which very useful for preparation of an affinity purified antigen.[18] The specific component which reacted with the MAb in that report was lipopolysaccharide, so the expression of the specific protein could not be done. The available reagents used in the serodiagnosis of septicemic melioidosis now are either crude[5-13] or purified antigen.[4,14] Both forms of antigen are directly prepared from the bacteria. Since ***B. pseudomallei*** is an hazardous agent and the mode of transmission is by inhalation, the specific protein purified by recombinant DNA technology will be safer than the conventional methods. Moreover, a large quantity of the specific antigen can be purified more easily without variation from lot to lot.

ACKNOWLEDGEMENTS

This work was partly supported by a grant from Srinakharinwirot University.

REFERENCES

1. Ashdown LR, Johnson RW, Koehler JM, Cooney CA. Enzyme-linked immunosorbent assay for the diagnosis of clinical and subclinical melioidosis. J Infect Dis 1989; 160 : 253-60.
2. Chaowagul W, White NJ, Dance DAB, *et al.* Melioidosis: a major cause of community-acquired septicemia in north-eastern Thailand. J Infect Dis 1989; 159 : 890-9.
3. Yee KC, Lee MK, Chua CT, Puthucheary SD. Melioidosis, the grate mimicker: a report of 10 cases from Malaysia. J Trop Med Hyg 1989; 91 : 249-54.
4. Anuntagool N, Rugdech P, Sirisinha S. Identification of specific antigens of *Pseudomonas pseudomallei* and evaluation of their efficiency for diagnosis of melioidosis. J Clin Microbiol 1993; 31 : 1232-6.
5. Alexander AD, Huxsoll DL, Warner A R Jr, Shepler V, Dorsey A. Serological diagnosis of human melioidosis with indirect hemagglutination and complement fixation tests. Appl Microbiol 1970; 20 : 825-33.

6. Appassakij H, Silpapojakul KR, Wansit R, Pornpakul M. Diagnostic value of the indirect hemagglutination test for melioidosis in an endemic area. Am J Trop Med Hyg 1990; 42 : 248-53.
7. Kunaphun P, Thirawattanasuk N, Suputtamonkol P, *et al.* Serology and carriage of *Pseudomonas pseudomallei*: a prospective study in 1000 hospitalized children in northeast Thailand. J Infect Dis 1993; 167 : 230-3.
8. Ashdown LR. Demonstration of human antibodies to *Pseudomonas pseudomallei* by indirect fluorescent antibody staining. Pathology 1981; 13 : 579-601.
9. Ashdown LR. Relationship and significance of specific immunoglobulin M antibody response in clinical and subclinical melioidosis. J Clin Microbiol 1981; 14 : 361-4.
10. Khupulsup K, Petchclai B. Application of indirect fluorescent antibody test for IgM antibody for diagnosis of melioidosis in Thailand. Am J Trop Med Hyg 1986; 35 : 366-9.
11. Kunakorn M, Boonma P, Khupulsup K, Petchclai B. Enzyme-linked immunosorbent assay for immunoglobulin M specific antibody for the diagnosis of melioidosis. J Clin Microbiol 1990; 28 : 1249-53.
12. Kunakorn M, Petchclai B, Khupulsup K, Naigowit P. Gold blot for detection of immunoglobulin M (IgM) and IgG specific antibodies for rapid serodiagnosis of melioidosis. J Clin Microbiol 1991; 29 : 2065-7.
13. Ismail G, Embi MN, Omar O, Allen JC, Smith CJ. A comparative immunosorbent assay for detection of *Pseudomonas pseudomallei* exotoxin. J Med Microbiol 1987; 23 : 353-7.
14. Petkanchanapong V, Naigowit P, Kondo E, Kanai K. Use of endotoxin antigens in enzyme-linked immunosorbent assay for the diagnosis of *P. pseudomallei* infection (Melioidosis). Asian Pac J Allergy Immunol 1992; 10 : 145-50.
15. Kearney JF. Hybridomas and monoclonal antibodies. In : Fundamental Immunology. Edited by Paul WE. New York : Raven Press 1984; 751-66.
16. Ashdown LR. Rapid differentiation of *Pseudomonas pseudomallei* from *Pseudomonas cepacia*. Lett Appl 1992; 14 : 203-3.
17. Pongsunk S, Sarasombath S, Ekpo P, Tangtherawattana P, Levine MM. Production of monoclonal antibodies to Vi polysaccharide antigen of *Salmonella typhi*. Asian Pac J Allergy Immunol 1993; 11 : 53-6.
18. Rugdech P, Anuntagool N, Sirisinha S. Monoclonal antibodies to *Pseudomonas pseudomallei* and their potential for diagnosis of melioidosis. Am J Trop Med Hyg 1995; 52(3) : 231-5.