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DIFFERENTIATION BETWEEN NON-VIRULENT AND VIRULENT *BURKHOLDERIA PSEUDOMALLEI* WITH MONOCLONAL ANTIBODIES TO THE ARA$^+$ OR ARA$^-$ BIOTYPES

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Abstract. *Burkholderia pseudomallei* is the causative agent of melioidosis, a fatal tropical infectious disease endemic in Southeast Asia. Environmental isolates of *B. pseudomallei* have two distinctive biotypes. Some soil isolates are arabinose-assimilators (Ara$^+$ biotype) and are non-virulent in experimental animals. The others cannot assimilate arabinose (Ara$^-$ biotype) and are virulent in experimental animals. The Ara$^-$ biotype is found in almost all *B. pseudomallei* clinical isolates. In the present study, a panel of eight monoclonal antibodies that agglutinate the bacteria were produced and tested. The first group, Bps-D2, -D3, -D5, -L1, and -L2 agglutinated 100% of Ara$^+$ clinical and soil isolates of *B. pseudomallei.* Another group Bps-A1, -A2, and -D1 agglutinated 92.9% and 90.9% of Ara$^-$ clinical and soil isolates, respectively. This panel of monoclonal antibodies may be useful for rapid differentiation between non-virulent Ara$^+$ and virulent Ara$^-$ *B. pseudomallei.*

INTRODUCTION

Burkholderia pseudomallei is the causative organism of melioidosis, a fatal tropical disease in humans and animals.[1] *Burkholderia pseudomallei* has been isolated throughout the tropics but infection of humans is particularly endemic in Southeast Asia and northern Australia.[2] In northeastern Thailand, *B. pseudomallei* is a major cause of morbidity and mortality in community-acquired septicemia.[3] The clinical course of septicemic melioidosis is often rapid deterioration with death within the first few days of hospitalization. The organism may also persist in the body for extensive periods and recrudesce years later. *Burkholderia pseudomallei* is a free-living saprophyte in soil and surface water, especially rice paddy fields.[4]

Previous surveys demonstrated that *B. pseudomallei* isolated from soil can be differentiated into two biotypes by the ability to assimilate L-arabinose.[5] The Ara$^-$ biotype (classical *B. pseudomallei*) is virulent in experimental animals and humans compared with the non-virulent Ara$^+$ biotype.[5,6] The latter has been proposed to be a new species named *Burkholderia thailandensis.*[7] Both biotypes are found in the soil in northeastern Thailand where melioidosis is endemic. In contrast, the Ara$^+$ biotype is found in other regions of Thailand where melioidosis is not endemic.[8] Both biotypes are morphologically similar and reported to be antigenically indistinguishable.[8] Our group has recently reported that the 16S ribosomal RNA gene sequence of the two biotypes is similar but distinguishable from each other by polymerase chain reaction (PCR) amplification using biotype-specific primers.[9] The ability to identify and classify the two biotypes in clinical and environmental isolates is useful to differentiate genuine clinical isolates from contaminants and also for the characterization of organisms in the soil.

MATERIALS AND METHODS

Bacterial isolates. A total of 237 isolates of *B. pseudomallei* were studied. One hundred and twenty-seven clinical isolates were obtained from patients in Khonkaen Regional Hospital and Siriraj Hospital. These isolates originated from blood, sputum, urine, pus, and other body fluids from patients with bacteriologically-proven melioidosis. All but one were of the Ara$^-$ biotype. One-hundred and ten isolates from soil were obtained from the collection of the Thailand Research Fund (TRF). All were identified as *B. pseudomallei* by the characteristic morphology of colonies on agar plates,[10] Gram stain, oxidase production, biochemical profiles, and resistance to gentamicin and colistin.[11] Arabinose assimilation was determined by growth on minimal-salt agar containing 0.2% L-arabinose as previously described.[12] Other bacteria (*Acinetobacter* spp., *Aeromonas* spp., *Burkholderia cepacia, Enterobacter* spp., *Escherichia coli, Haemophilus influenzae, Klebsiella* spp., *Proteus* spp., *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Ralstonia pickettii, Serratia* spp., *Stenotrophomonas maltophilia, Staphylococcus aureus, Streptococcus* group A, *Streptococcus* group B, *Streptococcus* group D, and *Streptococcus pneumoniae*) were from stock cultures maintained at the Laboratory of Cellular and Molecular Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Preparation of antigens and hybridoma production. *Burkholderia pseudomallei* isolates were cultured overnight at 37°C in brain heart infusion (BHI) broth (Difco, Detroit, MI). The bacterial suspension was fixed with 0.1% glutaraldehyde, sonicated and centrifuged at 8,000 × *g.* The supernatant fluid was used as the antigen for immunization. Hybridoma production was carried out by standard polyethylene glycol-mediated fusion of immune splenocytes and P3X63Ag8.653 myeloma cells.[13] Hybrid clones were selected by direct agglutination test with viable Ara$^+$ and Ara$^-$ *B. pseudomallei.* These were recloned by the limiting-dilution technique until monoclonality was obtained. Monoclonal antibodies were collected from culture supernatants and kept frozen at −20°C.

Characterization of the monoclonal antibodies. Reactivity and specificity of the monoclonal antibodies were tested by direct agglutination with Ara$^+$ and Ara$^-$ *B. pseudomallei,* and other medically-important gram-negative and gram-positive bacteria. Specificity was also evaluated by

TABLE 1
The reactivity profile of *Burkholderia pseudomallei*-specific monoclonal antibodies determined by a direct bacterial agglutination

Organism	MAbs/Isotype							
	Bps-A1 IgM	Bps-A2 IgM	Bps-D1 IgG	Bps-D2 IgM	Bps-D3 IgM	Bps-D5 IgM	Bps-L1 IgM	Bps-L2 IgM
Ara− *B. pseudomallei*	+	+	+	−	−	−	−	−
Ara+ *B. pseudomallei*	−	−	−	+	+	+	+	+
Other Gram-negative bacteria (14 species)	−	−	−	−	−	−	−	−
Gram-positive bacteria (5 species)	−	−	−	−	−	−	−	−

Western blot analysis. The isotypes of the monoclonal antibodies was determined using the ImmunoPure® Monoclonal Antibody Isotyping Kit I, as recommended by the manufacturer (Pierce, Rockford, IL).

SDS-PAGE and Western blot analysis. Whole bacterial lysates were mixed with sample buffer, heated and applied to gels. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA). Western blot was performed using monoclonal antibodies diluted 1:10 in 2% skimmed milk in Tris buffer. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD) (1:2,000 dilution in 2% skimmed milk in Tris buffer) were then added followed by alkaline phosphatase substrate. Positive reactions appeared as a purple band.

Agglutination test. Direct agglutination was evaluated by mixing 50 μl of the monoclonal antibodies with 10 μl of bacteria in a 96-well microtiter plate. Agglutination was observed under an inverted microscope (IMT-2, Olympus, Tokyo, Japan) at 100× magnification.

PCR amplification. The identity of each isolate was confirmed using PCR amplification of 16S rRNA gene with primers that specifically differentiate the Ara+ and Ara− biotypes of *B. pseudomallei,* as described previously.[9,14]

RESULTS

Reaction profile of *B. pseudomallei*-specific monoclonal antibodies. Eight hybridomas producing *B. pseudomallei*-specific monoclonal antibodies were obtained. All but one were IgM (Table 1). Each monoclonal antibody agglutinated either the Ara+ or Ara− *B. pseudomallei* isolates but not other bacteria (Table 1). The antibodies did not agglutinate any of the gram-positive or gram-negative bacteria tested. The monoclonal antibodies could be divided into 2 groups. The first, comprising Bps-D2, -D3, -D5, -L1, and -L2, agglutinated only Ara+ *B. pseudomallei* but not the Ara− biotype. On the other hand, the second group comprising Bps-A1, -A2, and -D1, agglutinated only the Ara− but not the Ara+ biotype. In addition, the antibodies agglutinated live *B. pseudomallei* as well as the glutaraldehyde-fixed bacterial preparations. They did not agglutinate *B. pseudomallei* heated at 70°C for 30 minutes. Therefore, the epitopes were likely to be surface antigen(s) that were heat-labile.

TABLE 2
Direct agglutination of clinical and soil isolates of Ara+ and Ara− *Burkholderia pseudomallei* by the monoclonal antibodies

B. pseudomallei		No. tested	Direct agglutination by monoclonal antibodies No. Positive (percent)	
Biotype	Source		Bps-D2, -D3, -D5, -L1, -L2	Bps-A1, -A2, -D1
Ara+	soil	55	55 (100)	0 (0)
	clinical	1	1 (100)	0 (0)
Ara−	soil	55	0 (0)	50 (90.9)
	clinical	126	0 (0)	117 (92.9)

Western blot analysis. The Bps-L1 and -L2 monoclonal antibodies were previously shown to react with lipopolysaccharide.[15] The other six monoclonal antibodies were not reactive on Western blot (data not shown).

Direct agglutination of Ara+ and Ara− clinical and soil isolates. One-hundred and twenty-seven clinical isolates and 110 soil isolates were evaluated by direct agglutination using the panel of eight monoclonal antibodies. Monoclonal antibodies Bps-D2, -D3, -D5, -L1, and -L2 could agglutinate 100% of Ara+ clinical and soil isolates of *B. pseudomallei* (Table 2). On the other hand, the monoclonal antibodies Bps-A1, -A2, and -D1 agglutinated 92.9% and 90.9% of Ara− clinical and soil isolates of *B. pseudomallei*, respectively. Nine Ara− clinical isolates and five Ara− soil isolates were not agglutinated by any of the monoclonal antibodies (Table 2).

To confirm the identity of Ara biotypes and the pattern of reactivity with the various monoclonal antibodies, 47 *B. pseudomallei* isolates were tested using multiplex PCR procedure that discriminated between Ara+ and Ara− *B. pseudomallei*.[9] Seventeen were confirmed to be the Ara+ biotype by both the biochemical test and PCR; all 17 were agglutinated by Ara+-specific antibodies. Thirty Ara− isolates, including 14 of that were not agglutinated by the monoclonal antibody panel and 16 that were agglutinated by the Ara−-specific monoclonal antibodies, were confirmed to be the Ara− biotype by both biochemical testing and PCR. Therefore, arabinose assimilation, PCR amplification of the 16S ribosomal RNA gene, and reactivities of the monoclonal antibodies were highly concordant for discrimination of the Ara+ and Ara− biotypes.

DISCUSSION

Soil isolates of *B. pseudomallei* include two biotypes. The major distinction is the difference in the ability to assimilate L-arabinose.[5] The Ara+ biotype is less virulent than the Ara− biotype (classical *B. pseudomallei*). In terms of their antigenic properties, these two biotypes were reported to be indistinguishable by reactivities with polyclonal antibodies or

antisera.[8] To date, monoclonal antibodies that specifically recognize Ara$^+$ but not Ara$^-$ biotype have not been described. Previous reports have only described monoclonal antibodies that specifically recognized the Ara$^-$ biotype, including one that recognized a high molecular weight component[16] and one specific for the bacterial exopolysaccharide.[17] Data in this study show that the Ara$^+$ and Ara$^-$ *B. pseudomallei* from soil and clinical isolates can be distinguished by a panel of monoclonal antibodies.

The Ara$^+$ biotype can be detected by direct agglutination using Ara$^+$-specific monoclonal antibodies Bps-D2, -D3, and -D5 as well as by the LPS-specific monoclonal antibodies Bps-L1 and -L2. The Ara$^-$ biotype can be detected by the Ara$^-$-specific monoclonal antibodies Bps-A1, -A2, and -D1. Using an LPS-specific monoclonal antibody Bps-L1 coated onto latex particles,[15] both biotypes can be detected by latex agglutination. Therefore, positive identification of *B. pseudomallei* and the Ara$^+$ and Ara$^-$ biotypes can be achieved in 100% and 90% of cases. Since monoclonal antibodies Bps-A1, -A2, -D1, -D2, -D3, and -D5 agglutinated either fresh or glutaraldehyde-fixed *B. pseudomallei* but not heat-treated organisms, it appears that these monoclonal antibodies recognize heat-labile surface antigens of the bacteria. However, the identity of the epitopes could not be defined by Western blot analysis using whole lysates of *B. pseudomallei.* Additional studies to identify the epitopes are underway.

Notably, all of the clones demonstrated high specificity against *B. pseudomallei.* They did not agglutinate 19 other species of other bacteria. This is likely due to the use of direct agglutination with viable *B. pseudomallei* as our selection criterion. Other monoclonal antibodies that do not distinguish the two biotypes and that cross-react with other bacteria have been obtained using similar protocols but different selection criteria.

Burkholderia pseudomallei lipopolysaccharide is similar among Ara$^-$ and Ara$^+$ isolates.[18] Interestingly, two of the monoclonal antibodies in this panel, Bps-L1 and -L2, recognized their lipopolysaccharide. These antibodies reacted with 97% of the Ara$^-$ and 100% of the Ara$^+$ *B. pseudomallei* isolates by Western blot and agglutination using antibody-coated latex particles.[15] However, Bps-L1 and -L2 lead to direct agglutination of the Ara$^+$ but not Ara$^-$ *B. pseudomallei.* This finding suggests that these two *B. pseudomallei* biotypes may be indistinguishable in terms of their lipopolysaccharides[18] but the epitopes recognized by these LPS-monoclonal antibodies expressed more prominently on the surface of the Ara$^+$ biotype.

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