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In Vitro and *In Vivo* Studies of Monoclonal Antibodies with Prominent Bactericidal Activity against *Burkholderia pseudomallei* and *Burkholderia mallei*▽

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Our laboratory has developed more than a hundred mouse monoclonal antibodies (MAbs) against *Burkholderia pseudomallei* and *Burkholderia mallei*. These antibodies have been categorized into different groups based on their specificities and the biochemical natures of their target antigens. The current study first examined the bactericidal activities of a number of these MAbs by an *in vitro* opsonic assay. Then, the *in vivo* protective efficacy of selected MAbs was evaluated using BALB/c mice challenged intranasally with a lethal dose of the bacteria. The opsonic assay using dimethyl sulfoxide-treated human HL-60 cells as phagocytes revealed that 19 out of 47 tested MAbs (40%) have prominent bactericidal activities against *B. pseudomallei* and/or *B. mallei*. Interestingly, all MAbs with strong opsonic activities are those with specificity against either the capsular polysaccharides (PS) or the lipopolysaccharides (LPS) of the bacteria. On the other hand, none of the MAbs reacting to bacterial proteins or glycoproteins showed prominent bactericidal activity. Further study revealed that the antigenic epitopes on either the capsular PS or LPS molecules were readily available for binding in intact bacteria, while the epitopes on proteins/glycoproteins were less accessible to the MAbs. Our *in vivo* study showed that four MAbs reactive to either the capsular PS or LPS were highly effective in protecting mice against lethal bacterial challenge. The result is compatible with that of our *in vitro* study. The MAbs with the highest protective efficacy are those reactive to either the capsular PS or LPS of the *Burkholderia* bacteria.

Burkholderia bacteria are a group of versatile Gram-negative bacteria. *Burkholderia pseudomallei* and *Burkholderia mallei*, two closely related species, are known to be highly pathogenic to human (25). *B. pseudomallei* is the causative agent of melioidosis, which is endemic in Southeast Asia and northern Australia (3, 19). The bacteria can apparently survive in a harsh environment for a long period of time. Its infection can be acquired through ingestion, inhalation, and direct contact. Clinically, melioidosis is a multifaceted disease and may present as an acute, subacute, or chronic infection, which eventually develops into the septicemic stage. The untreated septicemic melioidosis has a high mortality rate of 80% to 90%. Even with proper antibiotic treatment, the mortality rate still reaches 20% to 50% (3, 6, 12, 16, 27). It is very difficult to eradicate the bacteria in patients by using antibiotics. The melioidosis could relapse in 10 to 15% of the patients who had many years previously been "cured" with a prolonged period (20 weeks) of proper antibiotic treatment (16, 27). It has been reported that the dormant bacteria in the body cause the disease 10 years after the initial exposure (11). The mechanism of host-pathogen interaction for the bacteria is evidently quite unique.

B. mallei is the causative pathogen for glanders, another deadly multifaceted infectious disease (12, 26). This serious zoonotic disease primarily affects horses, mules, and donkeys. Although human disease is uncommon, it could be life-threatening and painful. Humans contract the disease by direct contact with skin exudates and respiratory secretion from infected equines, by ingestion of contaminated food, or by inhalation of bacterial dust. Without proper antibiotic treatment, the fatality rate of *B. mallei* infection can be as high as 95% (12). Cases of laboratory-acquired glanders through aerosols have been reported (7). Due to the ease of its transmission and the severity of illness it produces, *B. mallei* can be an obvious choice as a biological warfare agent or an agent for bioterrorism. In fact, *B. mallei* was used as a biological weapon in both World War I and II. Both *B. mallei* and *B. pseudomallei* have been classified as category B biothreat pathogens by the U.S. Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH). A widespread biological attack with either *B. pseudomallei* or *B. mallei* could have grave consequences to the world (12).

Presently, there are no effective vaccine and therapeutics available for these two pathogens. Therefore, more effective measures for the prevention and treatment of these diseases are urgently needed. Our laboratory has established numerous hybridoma cell lines derived from spleen cells of mice immunized with antigens prepared from several strains and clinical isolates of *B. pseudomallei* and *B. mallei* (9, 29). A total of 108 monoclonal antibodies (MAbs) that reacted strongly to *B.*

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pseudomallei and/or *B. mallei* had been characterized and categorized into 8 groups (from A to H). This classification was based on their binding patterns against a panel of 11 species of the *Burkholderia* bacteria and on the biochemical natures of the target antigens, such as lipopolysaccharides (LPS), capsular polysaccharides (PS), proteins, and glycoproteins, recognized by each MAb (9, 29). Some of these MAbs could potentially be developed into useful therapeutics in treating the devastating diseases caused by *B. mallei* and *B. pseudomallei*. In this study, we first examined the bactericidal activity of the MAbs chosen from different groups against *B. pseudomallei* and *B. mallei* by an *in vitro* opsonic assay by using differentiated HL-60 cells as phagocytes. We then studied the *in vivo* protective efficacy of selected MAbs against lethal challenge of *B. pseudomallei* and *B. mallei* in mice infected intranasally by the bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. pseudomallei* strain AFIP BP2 and *B. mallei* strain ATCC 23344 were used in this study; both bacterial cultures were obtained from the Armed Forces Institute of Pathology (AFIP) microbiology archive. The stock bacteria were inoculated onto nutrient agar plates prepared from nutrient broth powder and Bacto agar (BD Company, Franklin Lakes, NJ) and incubated at 37°C. To establish a bacterial growth curve, bacteria from a single colony were cultured overnight in nutrient broth. The bacteria were then diluted in the same medium to an optical density at 600 nm (OD_{600}) of 0.060 ± 0.005 and cultured for 11 h. The bacterial growth curve was established using a standard method (30).

Purification and quantification of MAbs. All MAbs against *B. pseudomallei* and *B. mallei* used in this study were produced in our laboratories as previously described (9, 29). Hybridoma cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and without antibiotics at 37°C. To produce MAbs, hybridoma cultures were inoculated at a concentration of 1 million CFU/ml in culture medium. The cells in the cultures were allowed to grow till extinction. The culture supernatants were harvested. Cells and cell debris were removed by centrifugation and by filtration through 0.22-um filter units. MAbs used for the *in vitro* studies were mainly in the form of supernatant derived from hybridoma cell cultures. Some MAbs used for *in vivo* study were purified from culture supernatants using an ÄKTAprime plus liquid chromatography system (GE Healthcare, Piscataway, NJ). In brief, the filtered culture supernatant was passed through a HiTrap rProtein A(G)-Fast Flow affinity column (GE Healthcare). The column was washed with 10× column volumes of equilibration buffer (20 mM sodium phosphate containing 150 mM NaCl, pH 7.5) and eluted with 100 mM glycine buffer (pH 3.0). The pH of the eluate was immediately neutralized to around pH 7.0 using 1 M Tris buffer (pH 10.0). The eluates from fraction tubes 1 to 4 (1 ml/tube) were pooled, concentrated, and then dialyzed using a Slide-A-Lyzer (10K molecular weight cutoff; Thermo Scientific, Rockford, IL). Binding properties against the respective antigens and the titer of purified MAbs were examined by enzyme-linked immunosorbent assay (ELISA) as previously described (9, 29). Purified MAbs were quantified by the ELISA using capture antibodies specific for each mouse immunoglobulin Fc subclass. Mouse immunoglobulin subclass standards used in the assay were purchased from Sigma-Aldrich, St. Louis, MO, and Bethyl Laboratories, Inc., Montgomery, TX.

Isotyping of MAbs. Isotyping of MAbs was done using the mouse MonAb-ID kit (Zymed Laboratories, South San Francisco, CA) by following the manufacturer's instructions. A similar MAb isotyping method was also established in our laboratories. Briefly, 96-well immunosorbent plates (Nunc-Immuno module 468667; Nalge Nunc International, Rochester, NY) were coated with specific goat anti-mouse immunoglobulin Fc subclass antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and blocked with 1% bovine serum albumin (BSA). Hybridoma culture supernatants or purified antibodies were added and incubated in the plates for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated detection antibodies specifically against each mouse immunoglobulin Fc subclass (Jackson ImmunoResearch Laboratories, West Grove, PA) were then added and incubated for 1 h. The isotype of each MAb was revealed using HRP substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; KPL, Gaithersburg, MD).

Bacterial killing assay. Overnight bacterial cultures with the absorbance at 600 nm (OD_{600}) around 0.7 for *B. mallei* and 1.2 for *B. pseudomallei* from a single colony were diluted 15-fold (*B. mallei*) or 30-fold (*B. pseudomallei*) with nutrient broth. The absorbance of the diluted bacteria cultures was about 0.05. The bacteria were cultured in a 37°C incubator for 4 h with shaking at 250 rpm to reach mid-log phase with OD_{600} around 0.5. Then, bacteria (3 ml in a 15-ml tube) were harvested by centrifugation at 2,000 × *g* for 30 min. The bacterial pellet was resuspended in phosphate-buffered saline (PBS). The OD_{600} of the bacterial suspension was adjusted to 0.25. The bacteria were then further diluted 10-fold with RPMI 1640 medium containing 5% heat-inactivated FBS. To determine the effects of MAbs against *B. mallei* and/or *B. pseudomallei* on bacterial growth, 50 μl of the above prepared bacteria (about 125,000 CFU) was inoculated into either nutrient broth or RPMI 1640 medium containing 5% heat-inactivated FBS. Then, MAbs (culture supernatants or purified antibody solutions adjusted to a final MAb concentration of 5 μg/ml) or control medium was added into the assay mixture. The final volume of the assay mixtures was 500 μl. The mixtures were incubated at 37°C with end-to-end rotation at 8 rpm for 0 h, 1 h, 2 h, 4 h, and 8 h. At the end of the incubation, the cultures were serial diluted. The diluted cultures were inoculated on nutrient agar plates and cultured in a 37°C incubator for 36 h (for *B. pseudomallei*) or 48 h (for *B. mallei*) for the CFU determination.

Induction of HL-60 cell differentiation. HL-60 (ATCC CCL-240) is a promyelocytic cell line originally derived from a patient with acute leukemia (4). These cells can differentiate into mature granulocytes with phagocytic function following the treatment with dimethyl sulfoxide (DMSO) (5). The differentiated HL-60 cells have been used in various opsonic studies (22). Before performing the opsonic assay, highly proliferating HL-60 cells were cultured in the presence of 1% DMSO for 6 days (23). The cells were then harvested and resuspended in RPMI 1640 medium containing 5% FBS at 4×10^6/ml.

Opsonophagocytosis assay. The opsonophagocytosis assay was performed following the procedures developed by Ho et al. with modifications (13). For phagocytes, we used differentiated HL-60 cells induced by DMSO instead of human peripheral polymorphonuclear leukocytes used in their tests. Briefly, 50 μl of each MAb solution (about 50 μg MAb/ml purified MAb or in hybridoma culture supernatants), 250 μl of differentiated HL-60 cell suspension, 50 μl of complement (20-fold-diluted guinea pig serum from Cedarlane Laboratories Ltd., Ontario, Canada), and 50 μl of *B. pseudomallei* or *B. mallei* suspension prepared in the same way as described above in the direct bacterial killing assay were mixed in a 1.5-ml screw-cap tube. Control tubes were set up by omitting one of these components. RPMI 1640 medium containing 5% heat-inactivated FBS was added to make the final volume 500 μl. The average ratio of differentiated HL-60 cells and target bacteria was around 1:1.25. The tubes were incubated at 37°C with end-to-end rotation at 8 rpm for 2 h, and then the mixtures were diluted 1,000-fold with H_2O containing 0.1% gelatin and kept at room temperature for 10 min in order to completely lyse the HL-60 cells. One hundred microliters of diluted samples was inoculated onto nutrient agar plates for CFU determination. Bacterial inhibitory activity was determined by the following formula: (number of CFU of bacteria cultured with phagocytes and complement only − number of CFU of bacteria cultured with MAb, phagocytes, and complement)/number of CFU of bacteria cultured with phagocytes and complement only × 100%.

Determination of binding of MAbs to bacterial preparation. Exponentially growing bacteria were inactivated by incubating in a 60°C water bath for 90 min. The bacterial cells were equally divided into two tubes, harvested by centrifugation, and washed with PBS six times. One of them was treated with PBS containing 2% sodium dodecyl sulfate (SDS), and the other tube was treated with PBS alone for 2 h at room temperature. The bacteria treated with PBS alone remained intact, while the bacteria treated with SDS were completely lysed. The resulting solutions were diluted with 50 mM sodium carbonate buffer, pH 9.6, and coated onto 96-well ELISA plates (1 μg bacterial protein in 100 μl/well). Antibody binding activities were determined by incubating 50 μl of hybridoma supernatant and detected by HRP-conjugated secondary antibodies specifically against individual subclass immunoglobulin Fc fragments of mouse antibodies. The plates were washed with PBS at each step, followed by color development with ABTS.

Animals and intranasal inoculation. Female BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratory (Madison, WI). Mice were maintained in cages (two to three per cage) which were placed in polystyrene microisolator units. Husbandry included a 12-h light-dark cycle, sterile cages and rodent chow, and water *ad libitum*. Before inoculation with bacteria, mice were anesthetized by intramuscular injection with a saline-diluted mixture of ketamine (100 mg/ml) at 0.15 mg/kg of body weight, acepromazine (5 mg/ml) at 30 mg/kg, and xylazine (20 mg/ml) at 3.5 mg/kg. The anesthetic mixture was administered

at a dose of 0.1 ml/kg of animal body weight. For intranasal inoculation, an aliquot of 25 μl of PBS containing the appropriate dose of the *Burkholderia* bacteria was administered into both nostrils, one at a time, with a micropipette tip. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Armed Forces Institute of Pathology. All bacterial culture preparation and storage as well as the animal challenge and maintenance were carried out in a biosafety level III facility at AFIP.

Determination of LD_{50}. Bacteria were prepared as described in the previous section. One day prior to the challenge, a single colony of bacteria was picked and grown in nutrient broth with shaking at 37°C overnight. The bacterial culture was then diluted 15- to 30-fold in fresh nutrient broth and incubated with shaking for 4 h to reach mid-log phase. Bacterial cells were harvested by centrifugation and washed with PBS. The bacterial pellet was then suspended in PBS to an OD_{600} reading of 0.25. To determine the 50% lethal dose (LD_{50}) of *B. pseudomallei* AFIP BP2 and *B. mallei* ATCC 23344 in mice, BALB/c mice were divided into groups of six. Five groups of mice were challenged intranasally with 5 different doses of bacteria. The targeted doses were 10, 100, 1,000, 10,000, and 100,000 CFU/mouse (the actual dose each group of mice received was adjusted after the numbers of CFU of each bacterial suspensions were determined following inoculation). Mortality was scored over 2 weeks. The LD_{50} was estimated for each experiment with the Reed-Muench method (20).

Passive protection assay. *B. pseudomallei* AFIP BP2 and *B. mallei* ATCC 23344 were prepared as described above and diluted to give an estimated challenge dose of 20-fold that of the LD_{50}. Groups of six mice under anaesthetization were intravenously injected with 100 μg of purified MAb in 0.4 ml of PBS or control serum. Mice were allowed to rest for 2 h prior to challenge intranasally with *B. pseudomallei* or *B. mallei*. The mice after challenge were observed daily, and mortality rate was recorded over a period of 3 weeks for *B. pseudomallei* and 2 weeks for *B. mallei*. The significance of the protective efficacy for each MAb was determined using Student's *t* test in Microsoft Excel by comparing the individual survival days of the six mice in each MAb-treated group to those in the normal-serum-treated control group.

RESULTS

Effect of MAbs on bacterial growth. To determine whether MAbs alone would affect the growth of *B. pseudomallei* or *B. mallei*, we simply added hybridoma supernatant or purified antibodies to the bacteria at a final MAb concentration of around 5 μg/ml in either nutrient broth or RPMI 1640 medium containing 5% FBS. The *B. pseudomallei* and *B. mallei* strains used for this study were the *B. mallei* ATCC 23344 strain and the *B. pseudomallei* AFIP BP2 strain. They were chosen because they had been used as the immunizing antigens for the hybridoma production and partially characterized by the MAbs in our previous study (29). *B. pseudomallei* and *B. mallei* were inoculated individually into each MAb preparation at a final density of 12,500 CFU/ml. Numbers of bacterial CFU at various time points between 0 to 8 h were determined. A total of 50 MAbs from group B, C, D, E, G, and H hybridomas were examined. Table 1 shows a list of the MAbs selected from each group and examined in this assay. Also included in this table are their isotypes and selected MAbs used in other assays performed in this study for the purpose of clarity. Specific features for each group of MAbs as described previously (29) are included and briefly summarized. In this assay, we found no significant difference in the numbers of CFU between cultures with MAbs and those without MAbs. It indicates that the specific MAbs alone do not affect bacterial growth.

Opsonic bactericidal effects of the MAbs. To determine whether our MAbs would promote killing of bacteria in the presence of phagocytic cells, we developed an opsonic assay to measure the change in numbers of viable bacteria. During the initial search for proper phagocytes to be used in our assay, we have evaluated phagocytes prepared from human peripheral blood, phagocytes prepared from mouse peritoneal cavity, and DMSO-induced differentiated human HL-60 cells (10, 22). It was found that differentiated HL-60 phagocytes by themselves did not have prominent bactericidal effects on *B. mallei* and *B. pseudomallei*. Undifferentiated HL-60 cells had little phagocytic function in the presence or absence of antibodies and complement. However, treatment with 1% DMSO for 5 to 6 days induced optimal differentiation of HL-60 cells into mature granulocytes (5). Figure 1 shows the changes in bacterial CFU between 0 and 2 h after treatment with different combinations of elements to evaluate the opsonic bactericidal effects of the MAbs. For the control, we examined the numbers of CFU in the presence of complement and HL-60 cells but without MAbs and in the presence of HL-60 cells alone. Without the addition of MAbs, bactericidal activity was minimal (no more than 20% reduction in the number of CFU compared to that of the culture with bacteria only, which is within the range of variation) in the presence of HL-60 cells alone or HL-60 cells plus complement.

For each MAb tested, we examined the role of complement in enhancing the opsonic bactericidal effects by comparing the number of CFU in cultures with complement to the number of CFU in cultures without complement. Our data clearly showed that the complement greatly enhanced the opsonic bactericidal effects of the MAbs. A preliminary study using different time points has shown that the bactericidal effects were optimal at 2 h after the addition of MAbs. The MAbs that led to a greater than 45% reduction in the number of CFU compared to the average number of CFU in controls were arbitrarily considered to have a strong opsonic activity. As shown in Fig. 1A, depicting results from one of the representative experiments, a total of 7 anti-*B. pseudomallei* MAbs from 4 different groups (B, E, G, and H) were measured for their opsonic bactericidal activity in reducing the viable number of bacteria. It is clearly shown that 4 (BP2 D2, BP7 2C6, BP7 1H7, and BP7 2G6) out of 7 tested MAbs had strong opsonic bactericidal activities against *B. pseudomallei*. Interestingly, among these 4 MAbs, 2 are from group B (anti-capsular PS of both *B. pseudomallei* and *B. mallei*) and 2 are from group E (anti-LPS of both *B. pseudomallei* and *Burkholderia thailandensis*), while the 3 MAbs from either group G (anti-glycoprotein of all *Burkholderia* species except for *B. thailandensis*) or group H (anti-protein of all *Burkholderia* species plus *Pseudomonas aeruginosa* and *Escherichia coli*) had little if any bactericidal effect. It suggests that MAbs against either capsular PS or LPS possess a greater opsonic activity than those against glycoproteins or proteins of the bacteria. Figure 1B shows the opsonic bactericidal activity of 5 MAbs from group C (anti-LPS of *B. mallei* only) and 1 from group D (anti-LPS of *B. mallei* with a low to moderate level of cross-reaction to *B. pseudomallei*). Again, 4 out of 5 group C MAbs (BML 11G6, BML 5D11, BML18F8, and BML 20F1) had shown strong bactericidal activities against *B. mallei*. The group D MAb (BMW2 11F2) had reduced the number of *B. mallei* CFU to 43% of that of the control average, which is slightly lower than what was defined as having a strong bactericidal activity. We have also compared the opsonic activity of BML 5D11and BML 20F1 in the form of purified antibodies to that of the culture supernatants in reducing the bacterial numbers. It showed little or no difference in the opsonic bactericidal activity against *B. mallei* between these two types of antibody samples.

Table 2 summarizes the results after examination of the

TABLE 1. List of MAbs and their isotypes used in all the assays performed in this study[a]

Group and MAb designation	Isotype	MAb examined by:			
		Direct effect on bacterial growth study	*In vitro* bactericidal study by opsonic assay	Antigen binding study by ELISA	*In vivo* passive protection study
Group B (anti-capsular PS MAbs of both *B. pseudomallei* and *B. mallei*)					
BP2 D2	IgG1(M)	√	√	√	–
BP2 D17	IgG1	√	√	–	–
BP A4	IgG2a	√	√	–	–
BP1 10F11	IgG3	√	√	√	–
BP72F4	IgG2b	√	√	√	√
BP7 3E10	IgG1	√	√	–	–
BP2 I67	IgG1	√	√	√	–
BP7 2C6	IgG2a	√	√	√	√
BMW5 1D9	IgM	√	√	–	–
BMW2 1B5	IgM	√	√	–	–
BP1 1E4	IgM	√	√	–	–
BP1 9G2	IgG3	√	√	√	–
BP1 6A8	IgM	√	√	√	–
BP1 2E7	IgG3	√	√	√	√
BP1 14A7	IgG3	√	√	√	–
BP7 1C10	IgM	√	√	√	–
BP7 7B2	IgM	√	√	√	–
BP1 14F4	IgM	√	√	–	–
BP 12G5	IgG3	√	√	–	–
BP7 4G9	IgM	√	√	–	–
BM1 12C5	IgG3	√	√	–	–
Group C (anti-LPS MAbs of *B. mallei* only)					
BMW 2D5	IgG1	√	√	–	–
BML8C8	IgG1	√	√	–	–
BML 11G6	IgG1	√	√	√	–
BML 5D11	IgG2b	√	√	√	√
BML 18F8	IgG1	√	√	√	√
BML 20F1	IgG1	√	√	√	–
BML 15G9	IgG1	√	√	√	–
BML 4C10	IgG1	√	√	√	–
BM13G12	IgG3	√	√	–	–
Group D (anti-LPS MAbs of *B. mallei* with low to moderate level of cross-reaction to *B. pseudomallei*)					
BMW2 11F2	IgG1	√	√	–	–
BMW2 14BC	IgG3	√	–	–	–
BMW2 3C8	IgG3	√	–	–	–
Group E (anti-LPS MAbs of both *B. pseudomallei* and *B. thailandensis*)					
BP7 2G6	IgG2a	√	√	√	√
BP1 7F7	IgG3	√	√	√	–
BP7 1H7	IgG1	√	√	√	–
BPL 5F7	IgG3	√	√	–	–
BPL 30D11	IgG2a	√	√	√	–
BP7 6G2	IgG2a	√	√	√	–
BP1 6G2	IgM	√	√	–	–
Group G (anti-glycoprotein MAbs of all *Burkholderia* species[b] except for *B. thailandensis*)					
BPL19B12	IgG1	√	√	√	–
BPL23E10	IgG2b	√	√	√	–
BML 9E2	IgG1	√	√	√	√
BMW5 5A1	IgM	√	√	√	–
BPL 17A2	IgG2b	√	√	√	–
BML 1F3	IgG1	√	√	–	–
BPL 23F11	IgG3	√	√	–	–
BP7 10B11	IgG1	√	√	√	√
Group H (anti-protein MAbs of all *Burkholderia* species plus *P. aeruginosa* and *E. coli*)					
BM2 17G6	IgM	√	√	√	–
BML 1F3	IgG1	√	√	√	–

[a] √, the particular MAb has been examined in the corresponding assay; –, not done.
[b] All *Burkholderia* species examined in our previous study include *B. cepacia* (ATCC 700070), *B. vietnamiensis* (ATCC BAA-248), *B. stabilis* (ATCC BAA-67), *B. ambifaria* (ATCC BAA-244), *B. caledonica* (ATCC BAA-462), *B. kururiensis* (ATCC 700977), *B. multivorans* (ATCC BAA-247), and *B. fungorum* (ATCC BAA-463).

opsonic bactericidal activity of a total of 47 MAbs from groups B, C, E, G, and H as listed in Table 1. In group B MAbs, which are specific for both *B. pseudomallei* and *B. mallei* capsular PS, 12 out of 21 (57%) MAbs tested against *B. pseudomallei* and 4 out of 10 (40%) MAbs tested against *B. mallei* showed strong opsonic bactericidal activity. In group C MAbs, which are specific for *B. mallei* LPS, 4 out of 9 (44%) MAbs tested against *B. mallei* showed strong opsonic bactericidal activity. Similarly, in group E MAbs, which are specific for *B. pseudomallei* and *B. thailandensis* LPS, 3 out of 7 (43%) tested against *B. pseu-*

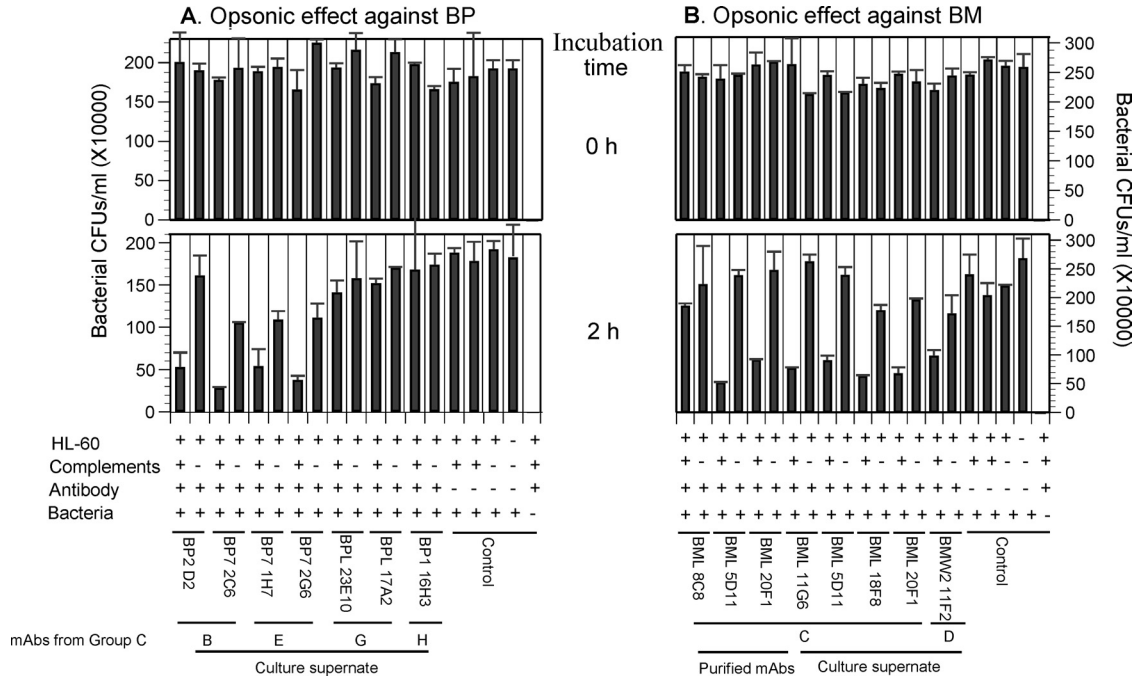

FIG. 1. Opsonic bactericidal activity of selected MAbs against *B. pseudomallei* (A) and *B. mallei* (B). Bacteria in log-phase growth were mixed with differentiated HL-60 cells and MAb in the presence or absence of complement in medium. At 0 and 2 h, CFU were determined for all cultures. Control cultures were set as without MAb, without both MAb and complement, and with bacteria only. Results shown are from two representative experiments.

domallei showed strong opsonic bactericidal activity. On the other hand, all 8 MAbs selected from group G or 2 from group H, specific for the bacterial glycoproteins or proteins, respectively, showed little if any opsonic bactericidal activity against either *B. pseudomallei* or *B. mallei*. Overall, our results showed that 15 out of 38 (39%) MAbs tested against *B. pseudomallei* and 8 out of 29 (28%) MAbs tested against *B. mallei* were found to have strong opsonic bactericidal activity. Table 3 lists the inhibitory activity of individual MAbs from groups B, C, and E with strong opsonic bactericidal activities against *B. pseudomallei* and *B. mallei*. Twelve MAbs from group B and three from group E demonstrated greater than 45% inhibitory activity against *B. pseudomallei* in the presence of complement. Four MAbs from group B and four from group C showed strong inhibitory activity against *B. mallei*. Among the group B MAbs tested, BP7 2C6, BP2 I67, BP2 2E7, and BP1 14A7 consistently demonstrated strong opsonic activity against both *B. mallei* and *B. pseudomallei*, with maximal inhibitory activities reaching more than 90%. Interestingly, sera from mice hyperimmunized with heat-killed *B. pseudomallei* or *B. mallei* did not show significant opsonic activity against either one of these two bacteria, although they do have strong binding activity as determined by ELISA (data not shown).

Immunoglobulin isotyping (as shown in Table 1) indicates that our MAbs are approximately 20% IgM and 80% IgG. Among the IgG MAbs there are IgG1, IgG2a, IgG2b, and IgG3 isotypes. Similar isotype distribution can also be found in the 19 MAbs with strong opsonic bactericidal activity. Four of them are IgM and 15 are IgG (six are IgG1, two are IgG2a, two are IgG2b, and five are IgG3). Therefore, the isotypes of the antibodies do not play a significant role in determining the strength of the opsonic effect.

Binding of MAbs to antigens on intact bacterial cells and in SDS lysates. Thus far, our results demonstrated that although

TABLE 2. The opsonic bactericidal activities of MAbs from different groups against *B. pseudomallei* and *B. mallei*

Group	Group characteristics (specificity and reactivity)	No. of MAbs tested for opsonic activity against[a]:			
		B. pseudomallei		*B. mallei*	
		Tested	With strong activity (%)	Tested	With strong activity (%)
B	Anti-*B. pseudomallei* and anti-*B. mallei* capsular PS	21	12 (57)	10	4 (40)
C	Anti-*B. mallei* LPS	ND		9	4 (44)
E	Anti-*B. pseudomallei* and anti-*B. thailandensis* LPS	7	3 (43)	ND	
G	Anti-glycoprotein of all *Burkholderia* species except for *B. thailandensis*	8	0 (0)	8	0 (0)
H	Anti-protein of all *Burkholderia* species plus *P. aeruginosa* and *E. coli*	2	0 (0)	2	0 (0)

[a] ND, not done.

TABLE 3. The inhibitory activity of individual MAbs against *B. pseudomallei* and *B. mallei*

MAb group	MAb identifier	% inhibitory activity (average ± SD) against[a]:	
		B. pseudomallei	*B. mallei*
B (anti-*B. pseudomallei* and anti-*B. mallei* capsular PS MAbs)	BP2 D2	60.54 ± 11.79	11.95 ± 8.02
	BP 14F4	53.3 ± 12.6	ND
	BP 12G5	45.7 ± 4	ND
	BP7 4G9	60.1 ± 6.8	ND
	BP7 2F4	56.92 ± 21.01	44.9 ± 2.2
	BP7 3E10	45.59 ± 14.85	21.33 ± 4.02
	BP2 I67	62.65 ± 15.76	60.37 ± 27.53
	BP7 2C6	71.75 ± 16.83	65.21 ± 23.60
	BP1 2E7	54.84 ± 19.72	71.07 ± 3.56
	BP1 14A7	45.16 ± 20.45	73.20 ± 5.27
	BP7 1C10	50.66 ± 12.08	4.2 ± 0.37
	BP1 9G2	53.06 ± 17.55	1.8 ± 0.74
E (anti-*B. pseudomallei* LPS MAbs)	BP7 2G6	69.81 ± 9.98	ND
	BP1 7F7	56.32 ± 16.66	ND
	BP7 1H7	51.99 ± 10.38	ND
C (anti-*B. mallei* LPS MAbs)	BML 11G6	ND	61.77 ± 9.60
	BML 5D11	ND	66.00 ± 9.95
	BML 18F8	ND	63.98 ± 15.01
	BML 20F1	ND	56.06 ± 17.92

[a] Data are calculated from multiple experiments (n = 2 to 8). ND, not done.

all our MAbs displayed strong antigen-binding activity by ELISA, they did not have equally strong *in vitro* opsonic bactericidal activity. All antibodies with strong opsonic bactericidal activity belonged to groups B, C, and E. None of them were from group G or H. The cause for this difference may be due to the location or accessibility of the antigens. To examine this possibility, binding activity against intact bacteria was compared to that against SDS-lysed bacteria by ELISA. As shown in Table 4, the binding activities of group B MAbs are mostly equally strong to either intact bacteria cells or the SDS lysates of *B. pseudomallei*, although SDS treatment seemed to slightly interfere with the binding of MAbs to their epitopes on *B. mallei* capsular PS. SDS treatment also made little change in binding to the LPS epitopes by group C and E MAbs. In contrast, while the binding activities of MAbs in groups G and H were weak to moderate against intact bacteria, there was a significant increase in the binding activity after the bacteria were lysed by SDS, which indicates that these proteins and glycoproteins are not located on the outer surface of the bacteria and will be released only after cell lysis.

Protective effects of anti-*Burkholderia* MAbs in challenged mice. To determine whether our MAbs could protect mice against lethal challenge of *B. pseudomallei* or *B. mallei*, an animal model, using BALB/c mice passively immunized with individual MAbs and then challenged intranasally with either *B. pseudomallei* or *B. mallei*, was established. The LD_{50} of *B. pseudomallei* AFIP BP2 and *B. mallei* ATCC 23344 in mice infected through intranasal inoculation was first estimated by the Reed-Muench method (20). The LD_{50} was estimated to be 27 CFU for the *B. pseudomallei* AFIP BP2 strain and 8,000 CFU for the *B. mallei* ATCC 23344 strain. To examine the protective effects of MAbs against *B. pseudomallei*, mice received individual MAbs through intravenous injection and were then challenged intranasally with *B. pseudomallei* at CFU numbers 20× that of the LD_{50}. Mice of the control group received normal mouse serum. To examine whether MAbs with strong *in vitro* opsonic bactericidal activity also provide protection against infection in animals, five MAbs selected from three different groups (BP7 2C6 and BP7 2F4 from group B, BP7 2G6 from group E, BP7 10B11 and BML 9E2 from group G) were evaluated for their protective efficacy against lethal *B. pseudomallei* challenge. Results showed that while mice in the control group had an 80% mortality rate (5 out of 6 mice died) (Fig. 2), groups of mice that received MAb BP7 2C6 (group B MAbs) and MAb BP7 2G6 (group E MAbs) were completely protected during the 3-week observation period (Fig. 2). Comparing the individual survival times of the mice in either the MAb BP7 2C6- or MAb BP7 2G6-treated group to those of the control serum-treated group within the 3-week observing period showed a significant difference between the MAb-treated group and the control group ($P < 0.005$). On the other hand, mice treated with either MAbs BP7 10B11 or BML 9E2 (both are group G MAbs) had a mortality rate of 67% (8 out of a total of 12 mice died), which showed no significant difference from that of the control group. Another group B antibody, MAb BP7 2F4, gave partial protection with a 33% mortality rate after *B. pseudomallei* challenge ($P < 0.05$). To examine the passive protection of MAbs against *B. mallei* ATCC 23344 challenge, four MAbs against *B. mallei* selected from groups B (BP1 2E7 and BP7 2F4) and C (BML 5D11 and BML 18F8) were evaluated. The results showed that mice that received either MAb BP1 2E7 or MAb BP7 2F4 (both are group B MAbs) completely survived *B. mallei* challenge through a period of 14 days, in contrast to mice in the control group that received normal serum, which all died within 5 days after challenge ($P < 0.001$) (Fig. 3). Two groups of mice that received either MAb BML 5D11 or BML 18F8 (group C MAbs) showed a mortality rate of 17% ($P < 0.001$) and 50% ($P < 0.05$). In summary, we selected a total of eight MAbs against *B. pseudomallei* and/or *B. mallei* to study their passive protection efficacy against lethal challenge in BALB/c mice. It was found

TABLE 4. Change in binding activities of MAbs between intact *B. pseudomallei* and *B. mallei* cells and their SDS lysates

MAb	Isotype	Binding activity of MAb[a]					
		B. mallei 23344			*B. pseudomallei* AFIP BP2		
		Intact cells	SDS lysate	Change	Intact cells	SDS lysate	Change
Group B (anti-*B. pseudomallei* and anti-*B. mallei* capsular PS MAbs)							
BP2 D2	IgM	++++	++++		++++	++++	
BP1 10F11	IgG3	++++	++++		++++	++++	
BP7 2F4	IgG2b	+++	++	↓	+++	+++	
BP2 I67	IgG1	++++	+++	↓	++++	++++	
BP7 2C6	IgG2a	++++	+++	↓	++++	++++	
BP1 9G2	IgG3	++++	++++		++++	++++	
BP1 6A8	IgM	++++	++++		++++	++++	
BP1 2E7	IgG3	+++	+++		++++	+++	↓
BP1 14A7	IgG3	++++	+++	↓	++++	++++	
BP7 1C10	IgM	++++	++++		++++	++++	
BP7 7B2	IgM	++++	++++		++++	++++	
Group C (anti-*B. mallei* LPS MAbs)							
BML 11G6	IgG1	++++	++++				
BML 5D11	IgG2b	++++	++++				
BML 18F8	IgG1	++++	++++				
BML 20F1	IgG1	+++	++++	↑			
BML 15G9	IgG1	++++	++++				
BML 4C10	IgG1	++++	++++				
Group E (anti-*B. pseudomallei* LPS MAbs)							
BP7 2G6	IgG2a				++++	**++++**	↑
BP1 7F7	IgG3				+++	+++	
BP7 1H7	IgG1				++++	++++	
BPL 30D11	IgG2a				++++	++++	
BP7 6G2	IgG2a				++++	++++	
Group G (anti-glycoprotein MAbs)							
BPL19B12	IgG1	++	++++	↑ ↑	+	++++	↑ ↑
BPL23E10	IgG2b	+++	++++	↑	++	++++	↑ ↑
BML 9E2	IgG1	++	++++	↑ ↑	+	++++	↑ ↑
BMW5 5A1	IgM	+	++++	↑ ↑	+	++	↑
BPL 17A2	IgG2b	+++	++++	↑	++	++++	↑ ↑
BP7 10B11	IgG1	+++	++++	↑	+++	++++	↑
Group H (anti-protein MAbs)							
BML 1F3	IgG1	+	++++	↑ ↑	+	+++	↑ ↑
BM2 17G6	IgM	+	++	↑	+	+	

[a] +, weak activity, OD reading between 0.1 and 0.5; ++, moderate activity, OD reading between 0.5 and 1.0; +++, strong activity, OD reading between 1.0 and 2.0; ++++, very strong activity, reading higher than 2.0; ↑, increase in binding activity; ↑ ↑, higher increase in binding activity; ↓, decrease in binding activity; blank cell, no change. Experiments were repeated at least three times.

that MAbs BP7 2C6 and BP7 2G6 showed complete protection against *B. pseudomallei* challenge and MAbs BP 2E7 and BP 2F4 showed complete protection against *B. mallei* challenge within our observation periods. These MAbs are specifically against either capsular PS or LPS, which demonstrated a consistent pattern to our *in vitro* bactericidal study in that MAbs that belong to groups B, C, and E are more effective in bacteria killing and therefore are more protective in our animal study than those of groups G and H.

DISCUSSION

B. mallei and *B. pseudomallei* are two highly pathogenic species of bacteria to human. The mortality rate of patients with melioidosis and glanders following *B. pseudomallei* and *B. mallei* infections remains very high, despite aggressive antibiotic treatments. The mechanisms of host-pathogen interaction for *Burkholderia* bacteria are evidently unique. The immunity developed from the disease or repeated exposure to *B. pseudomallei* seemed insufficient to prevent a relapse or to provoke a protective response for primary infection (3). Development of various approaches of immunotherapy has been attempted (2, 8, 12). Adoptive transfer of mononuclear leukocytes from *B. pseudomallei*-immunized mice was unable to provide protection against a lethal challenge of the bacteria (1). On the other hand, *B. mallei*- and *B. pseudomallei*-specific MAbs were reported to be effective in protecting the animals against lethal challenges of the bacteria (13, 15, 24). Our laboratory has developed more than 100 MAbs against *Burkholderia* bacteria. In an effort to search for potent therapeutic MAbs, we first examined the *in vitro* bactericidal effects of these MAbs. It has shown that MAbs alone did not affect bacterial growth; how-

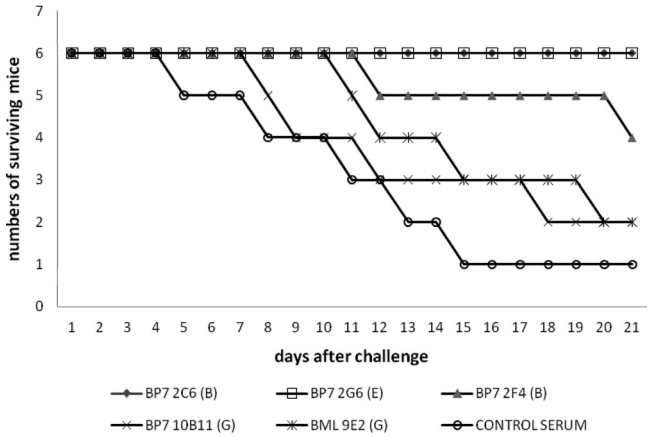

FIG. 2. Passive MAb protection against intranasal *B. pseudomallei* AFIP BP2 challenge in mice. Six groups of mice (6 mice per group) received intravenously normal serum (with the amount of mouse IgG equal to 100 μg) or one of 5 purified MAbs (100 μg Ig per mouse). Mice rested for 2 h and then were challenged intranasally with 20× the number of the LD_{50} CFU of *B. pseudomallei*. Mortality rate was scored over a period of 3 weeks.

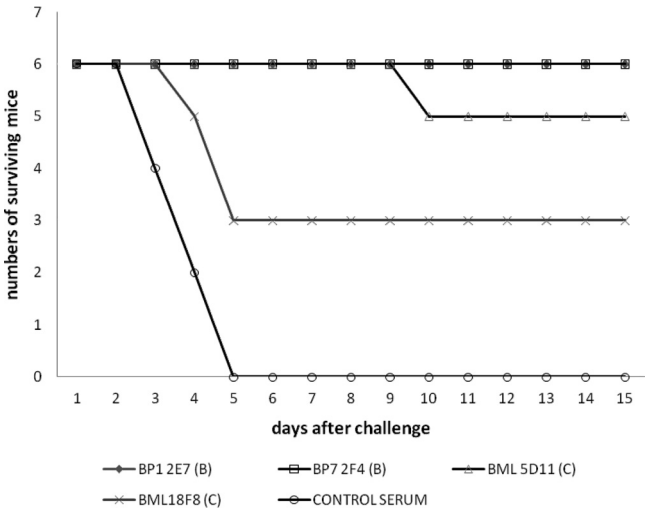

FIG. 3. Passive MAb protection against intranasal *B. mallei* ATCC 23344 challenge in mice. Five groups of mice (6 mice per group) received intravenously normal mouse serum or one of four purified MAbs (100 μg Ig per mouse). Mice rested for 2 h and then were challenged intranasally with 20× the number of the LD_{50} CFU of *B. mallei*. Mortality rate was scored over a period of 15 days.

ever, in the presence of complement and phagocytes, a number of the selected MAbs possessed strong bactericidal effects. The differentiated HL-60 phagocytes have been widely used by many researchers in different opsonic assays (10, 22). However, variations in the opsonic bactericidal activities among experiments did occur occasionally in our study. Multiple repeats for these experiments were employed to reduce the range of standard deviations.

It is important to note that normal serum (from either human, rabbit, or guinea pig) *per se* added at a higher concentration could produce a significant inhibitory effect on the bacteria, especially on *B. mallei*. This kind of bactericidal effect had been suggested as the result of complement and low-binding-affinity, broad-spectrum natural antibodies present in these sera (14, 17, 21, 28). Heat inactivation of the sera at 56°C for 30 min effectively abolished the bactericidal effect of these sera, indicating that complement was involved. In our present study, we selected guinea pig serum as the source of complement. The serum sample was first diluted 20-fold before being added to the mixture of opsonic assay solution. The final concentration of guinea pig serum in the assay was 0.5%. At this concentration, the guinea pig serum alone had no inhibitory effect on the growth of *Burkholderia* bacteria, but it provided sufficient complement to mediate the bactericidal effects of MAbs in the presence of phagocytes.

Our previous study revealed that most MAb groups are able to recognize more than one species among the *Burkholderia* genus, except for the group C MAbs, which recognize only *B. mallei* (29). The present study showed that the opsonic bactericidal activities varied significantly not only among different groups of MAbs but also among MAbs in the same group. This significant variation of antibacterial effects among MAbs in the same antibody group is most likely due to differences in the locations of their respective epitopes present on the same antigens and in their antigen-binding affinities. A surface plasmon resonance (SPR) biosensor (Biacore) is presently employed to study the binding interactions between these MAbs and their respective antigens to evaluate these two possibilities (18). All MAbs found to possess strong opsonic bactericidal activities were in the groups of B, C, and E of the initial classification. Our ELISA demonstrated that the location of these antigens/epitopes played an essential role in determining the effectiveness of the opsonic activity of the MAbs. Capsular PS and LPS on intact bacteria are apparently more accessible to the MAbs than the proteins and glycoproteins. The accessibility of the target antigens on intact bacteria to the MAbs should be prerequisite to antibody-mediated phagocytosis. As a result, MAbs against intracellular antigens are not suitable for antibody-mediated phagocytosis.

It is interesting to note that antisera from the mice hyperimmunized with heat-killed *B. mallei* and *B. pseudomallei*, in comparison with many *B. pseudomallei*- and *B. mallei*-specific MAbs, produced only weak opsonic antibacterial effects in our assay. One possibility could be that the amount of anti-capsular PS and LPS antibodies in the serum samples are too low to offer strong bactericidal effects under our assay condition since antisera also contain numerous antibodies against glycoproteins/proteins.

Isotyping of the MAbs reveals another interesting finding: the isotypes of the MAbs do not affect their opsonic activity. There are two types of receptors present on the phagocytes that mediate opsonophagocytosis. One is the Fc receptor, and the other one is the complement receptor. HL-60 cells have previously been shown to express both receptors after induction to differentiate (10). As discussed earlier, under our experimental condition, the concentration of complements was too low to opsonize the *B. mallei* or *B. pseudomallei* bacteria. MAbs of various isotypes alone activated opsonophagocytosis only moderately. It is in accordance with the fact that Fc receptors, such as FcγR II on the HL-60 cells, are low-affinity receptors for IgG1, IgG2, and IgG3 (10). However, in the presence of both, complement and antibodies synergistically

opsonized the bacteria, leading to a much greater extent of phagocytosis by the HL-60 cells. Therefore, binding of the MAb onto bacteria can further fix the complements onto the antibody-bacteria immune complex, resulting in a much stronger binding to the HL-60 cells and a more effective elimination of the bacteria.

Our *in vivo* protective study showed consistent results to our *in vitro* bactericidal study, which indicates that the opsonic bactericidal assay can provide valuable information in determining the effectiveness of the MAbs to confer passive immune protection against intranasal *B. pseudomallei* and *B. mallei* challenge. Both capsular PS and LPS were known to be important virulence factors in bacterial infection. Trevino et al. have shown that anti-LPS MAbs are effective in protecting mice from lethal aerosol challenge of *B. mallei* (24). Our studies further showed that in addition to anti-LPS MAbs, anti-capsular PS MAbs were even more effective in early protection against intranasal challenge of *B. mallei*. Previous studies by Jones et al. have shown that a cocktail of mixed MAbs against exopolysaccharide, LPS, and proteins demonstrated a significant protective effect against intraperitoneal challenge of *B. pseudomallei* (15). Compared individually, both anti-exopolysaccharide and anti-LPS MAbs performed better in protection than the anti-protein MAbs. Our studies are in agreement with their study and further showed that the location of the antigens plays an important role in determining the effectiveness of the antibodies. Our studies also showed that not all anti-capsular PS or LPS MAbs are equally effective. As mentioned earlier, the location of each epitope and the affinity between the antibodies and their respective epitopes may affect their ability in performing opsonophagocytosis and complement fixation. It is known that the aerosol route can induce a much more acute and severe infection than the intraperitoneal route and therefore is more difficult to combat. Our MAbs were shown to provide protection against intranasal challenge of *B. pseudomallei* for at least 3 weeks and *B. mallei* for 2 weeks. This pioneer study suggests that our model may be suitable for searching MAbs with high efficiency in combating *B. pseudomallei* and *B. mallei*. A more comprehensive and thorough animal study will ensue to show the effectiveness of these MAbs in stopping or deterring melioidosis and glanders in the long run. Furthermore, these MAbs with high performance in anti-*B. pseudomallei* and anti-*B. mallei* activities could potentially be genetically modified and developed into effective therapeutics in prevention or treatment against infections by these two highly pathogenic bacteria in human.

In conclusion, we have examined bactericidal activity of a large panel of MAbs specific to various antigens of *B. pseudomallei* and *B. mallei* by *in vitro* opsonic assay and found that MAbs with strong opsonic activities are those with specificity against either the capsular PS or LPS of the bacteria. Several MAbs were selected to be evaluated for their effects on passive protection against *Burkholderia* infection in BALB/c mice by intranasal challenge with a lethal dose of bacteria. It was again found that the MAbs with the highest protective efficacy are those reactive to either the capsular PS or LPS.

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