Monoclonal Antibodies Passively Protect BALB/c Mice against *Burkholderia mallei* Aerosol Challenge

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Glanders is a debilitating disease with no vaccine available. Murine monoclonal antibodies were produced against *Burkholderia mallei*, the etiologic agent of glanders, and were shown to be effective in passively protecting mice against a lethal aerosol challenge. The antibodies appeared to target lipopolysaccharide. Humoral antibodies may be important for immune protection against *B. mallei* infection.

Glanders, a disease caused by the microorganism *Burkholderia mallei*, was originally described around 425 B.C. and has been a world problem throughout most of recorded history. Humans can acquire the disease from infected animals by inhalation of the organism or through breaks in the skin. Human disease has been described as "loathsome" (15) and is characterized by purulent abscesses in infected tissues and organs (12). While *B. mallei* has seemingly been eradicated from many parts of the world today, its classification as a category B biothreat agent suggests that the microorganism could have a significant negative worldwide impact on human health. No vaccines against glanders exist, and current diagnostic tests cannot discriminate between infections caused by *B. mallei* and those caused by *Burkholderia pseudomallei*, another category B pathogen (8). Antibiotic therapy is usually successful with timely diagnosis of the disease.

The relative importance of humoral antibodies and cell-mediated immune responses in glanders immunity remains to be elucidated. Attempts to passively transfer immunity with immune serum were unsuccessful (10, 17). Published reports appear to suggest a greater role for cell-mediated immunity than humoral immunity in the limitation of the early spread of *B. mallei* in the host (5, 6). Irradiated *B. mallei* induced a Th1- and Th2-like cytokine response and a Th2-like subclass immunoglobulin response in BALB/c mice (1). Interleukin-12 (IL-12) has been shown to enhance protective immunity against glanders. Vaccinating mice with irradiated *B. mallei* plus IL-12, but not irradiated *B. mallei* alone, appears to induce partial protective immunity against a lethal subcutaneous challenge with *B. mallei* (K. Amemiya, personal communication). Injecting plasmid DNA encoding IL-12 in mice to induce in vivo expression of the cytokine resulted in a significant protection against aerosol infection with *B. mallei* (R. Ulrich, personal communication). An attenuated branched-chain amino acid auxotroph mutant of *B. mallei* was recently shown to partially protect against aerosol infection in mice (16).

In an attempt to generate specific antibodies that could be useful in the specific diagnosis of glanders and in prevention of disease, monoclonal antibodies (MAbs) were generated in BALB/c mice. In determinations of antibody specificity or efficacy against infection, results were evaluated for statistical significance by linear regression analysis or by analysis of variance. Survival distributions were compared by Kaplan-Meier methods. All tests were at the 95% confidence level (two tailed) (14).

To generate monoclonal antibodies, mice were injected intraperitoneally (i.p.) with 100 µg of irradiation-killed mid-log-phase *B. mallei* China 7 strain (ATCC 23344) cells and given a second injection 14 days later. Three days after the booster injection, a splenocyte suspension was prepared from the mouse with the highest enzyme-linked immunosorbent assay titer against *B. mallei* and fused with the murine myeloma cell line P3X63-Ag8.653 (7). Primary hybridoma culture supernatants were screened for antibody activity by enzyme-linked immunosorbent assay with irradiated *B. mallei*- or *B. pseudomallei*-coated microtiter wells, and *B. mallei*-positive hybridomas were subcloned by limiting dilution. Thirty-two clones reacted with *B. mallei* antigens and were selected for further specificity screening (data not shown). Of these, four anti-*B. mallei* clones were finally selected, based on their strong reactivity with *B. mallei* and absence of reactivity with the closely related *B. pseudomallei* (8). Antibodies were purified by protein A chromatography; purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Culture supernatants from the four selected hybridomas, designated 1G2-1D3, 1G3-1, 9C1-2, and 8G3-1B11, reacted with *B. mallei* even at high dilutions of culture supernatants tested (Table 1). At each dilution tested, absorbencies of microtiter wells coated with *B. mallei* antigens were significantly greater ($P < 0.003$) than absorbencies of microtiter wells coated with *B. pseudomallei* antigens.

To determine the ability of the anti-*B. mallei* MAbs to specifically capture *B. mallei* antigens in solution, *B. mallei* or *B. pseudomallei* cell lysates were added to microtiter wells precoated with individual anti-*B. mallei* MAb. Antigen capture was detected by adding a heterologous anti-*B. mallei* MAb conjugated to biotin (Table 2). All four anti-*B. mallei* MAbs

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14. ABSTRACT

Glanders is a debilitating disease with no vaccine available. Murine monoclonal antibodies were produced against Burkholderia mallei, the etiologic agent of glanders, and were shown to be effective in passively protecting mice against a lethal aerosol challenge. The antibodies appeared to target lipopolysaccharide. Humoral antibodies may be important for immune protection against B. mallei infection.

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TABLE 1. Specificity of anti-*B. mallei* monoclonal antibodies

MAb	Isotype	Absorbance at a hybridoma culture supernatant dilution of:					
		64	128	256	512	1,024	2,084
8G3-1B11	IgG2a	1.40[a]	1.12	0.82	0.52	0.31	0.18
		0.15[b]	0.09	0.06	0.04	0.03	0.03
9C1-2	IgG2b	0.86	0.88	0.72	0.59	0.43	0.49
		0.11	0.09	0.11	0.10	0.10	0.16
1G2-1D3	IgG2a	1.17	0.82	0.55	0.35	0.20	0.14
		0.03	0.03	0.03	0.02	0.02	0.02
1G3-1	IgG2a	1.39	1.29	0.89	0.67	0.42	0.38
		0.14	0.13	0.11	0.11	0.10	0.11

[a] Absorbance obtained with microtiter wells coated with *B. mallei* antigens.
[b] Absorbance obtained with microtiter wells coated with *B. pseudomallei* antigens.

TABLE 2. Specific antigen capture by anti-*B. mallei* monoclonal antibodies

Monoclonal antibody coat	Bacterial antigen added[a] (μg/ml)					
	Burkholderia mallei			Burkholderia pseudomallei		
	5.0	10.0	20.0	5.0	10.0	20.0
8G3-1B11	0.450[b]	0.686	0.827	0.120	0.127	0.113
1G2-1D3	0.349	0.424	0.603	0.137	0.141	0.140
1G3-1	0.351	0.450	0.698	0.110	0.116	0.118
9C1-2	0.481	0.663	0.910	0.136	0.106	0.099
F1-04-A-G1	0.110	0.112	0.178	0.112	0.118	0.145

[a] *B. mallei* or *B. pseudomallei* antigens (100 μl) were added to microtiter wells precoated with various anti-*B. mallei* MAbs (50 μl of 20 μg/ml).
[b] Average absorbance of triplicate determinations.

were capable of capturing *B. mallei*, but not *B. pseudomallei*, antigens in solution ($P < 0.0001$). The negative control MAb termed F1-04-A-G1, specific for the F1 capsular antigen of *Yersinia pestis* (2), did not bind to either of the two *Burkholderia* cell lysates ($P > 0.05$). The ability of the MAbs to specifically recognize *B. mallei* and not the closely related *B. pseudomallei* antigens in solid phase, as well as in solution, suggests their potential value as specific diagnostic tools in differentiating glanders and melioidosis in clinical conditions.

We then determined the antigenic specificity of the four anti-*B. mallei* MAbs by immunoblot analysis of *B. mallei* separated by SDS-PAGE with 10 to 20% precast Tricine gels (Invitrogen) (11). The immunoblot reactivity of two representative MAbs (8G3-1B11 and 1G2-1D3) is shown in Fig. 1a. Both MAbs reacted with *B. mallei* antigens characterized by a typical LPS ladder-banding pattern, ranging from approximately 20 kDa to 50 kDa in molecular mass (Fig. 1a, lanes 1 and 2). No reactivity was detected in immunoblot analyses of irradiated *B. pseudomallei* separated by SDS-PAGE (data not shown). Pretreating the irradiated *B. mallei* bacterial lysate with proteinase K did not abolish nor alter the immunoblot reactivity or the typical ladder-banding pattern, further supporting the lipopolysaccharide (LPS) nature of the antigens recognized (Fig. 1b, lane 2). Again, no immunoblot reactivity was evident with irradiated *B. pseudomallei* bacterial lysate (Fig. 1b, lane 1). Ample experimental evidence exists to suggest an important role of bacterial capsules in the virulence of both gram-negative and gram-positive pathogens. The capsular exopolysaccharide of *B. mallei* and *B. pseudomallei* was recently demonstrated to play a vital role in enhancing the virulence of the bacteria (4, 13). Whether or not the anti-*B. mallei* MAbs described herein are specific for LPS associated with the bacterial capsule remains to be determined.

We were interested in determining whether our MAbs could passively protect mice against a lethal aerosol challenge of *B. mallei*. Groups of six BALB/c mice each were injected i.p. with 1.0-mg/mouse of purified anti-*B. mallei* MAbs and challenged by whole-body aerosol 18 h thereafter with 20 50% lethal doses (LD$_{50}$; 1.9×10^4 CFU) of an overnight culture in mid-log phase of *B. mallei*, strain China 7 (ATCC 23344). Negative control groups were injected with either Hanks' balanced salt solution or MAb F1-04-A-G1 specific for the F1 antigen of *Y. pestis*. After challenge, the mice were observed daily, and mortalities

and the mean time to death were recorded. At the end of the experiment, all survivors were humanely killed. Immunization and challenge experiments were conducted in biosafety level 3 (BSL3) and BSL4 environments, respectively. As expected, the negative control F1-04-A-G1 did not protect the mice from the challenge (Fig. 2). Conversely, the anti-*B. mallei* MAbs provided significant protection. Monoclonal antibody 1G2-1D3 completely protected the mice against aerosol challenge (100% survival). Significant protection was also observed in mice treated with MAbs 9C1-2 and 1G3-1 (83% survival). All protected mice survived up to 14 days postchallenge, at which time the experiment was terminated. There were no significant differences in the ability of the MAbs to protect from death ($P > 0.05$). All MAbs were able to significantly protect against death ($P < 0.01$), compared to the irrelevant MAb control (F1-04-A-G1).

To assess the immunotherapeutic potential of the anti-*B. mallei* MAbs, groups of six mice each were treated with 1 mg of 1G2-1D3 anti-*B. mallei* MAb either 18 h before or 18 h after aerosol challenge. Treatment with 1G2-1D3 MAb 18 h before challenge again completely protected the mice against a lethal *B. mallei* aerosol challenge ($P < 0.001$) (Fig. 3). However, this anti-*B. mallei* MAb administered 18 h postchallenge was ineffective in providing protection ($P > 0.05$). All mice in this group, as well as those in the negative MAb (F1-04-A-G1) control group, died 4 days postchallenge.

This represents, to our knowledge, the first demonstration of passive protection against *B. mallei* infection in a murine model of aerosolized glanders by MAbs. Note that although significant passive protection was achieved, the spleens of all surviving mice killed at the end of the experiment contained significant numbers of *B. mallei* cells. Thus, the immune protection mediated by anti-*B. mallei* MAbs was not sterile but appeared to result from an antibody-induced reduction of the number of infecting pathogens below the lethal threshold. Whether antibody administered <18 h postchallenge would provide protection remains to be determined. It is clear that antibody-mediated protection is most effective during the initial phase of infection, presumably by clearing the pathogens from the circulation and/or limiting or reducing the number of pathogens being internalized by the primary host target cells, the macrophages. A likely explanation for the lack of protection by MAb treatment administered 18 h postchallenge is that once phagocytosed, the intracellular pathogens are protected from and therefore less affected by circulating antibodies, at
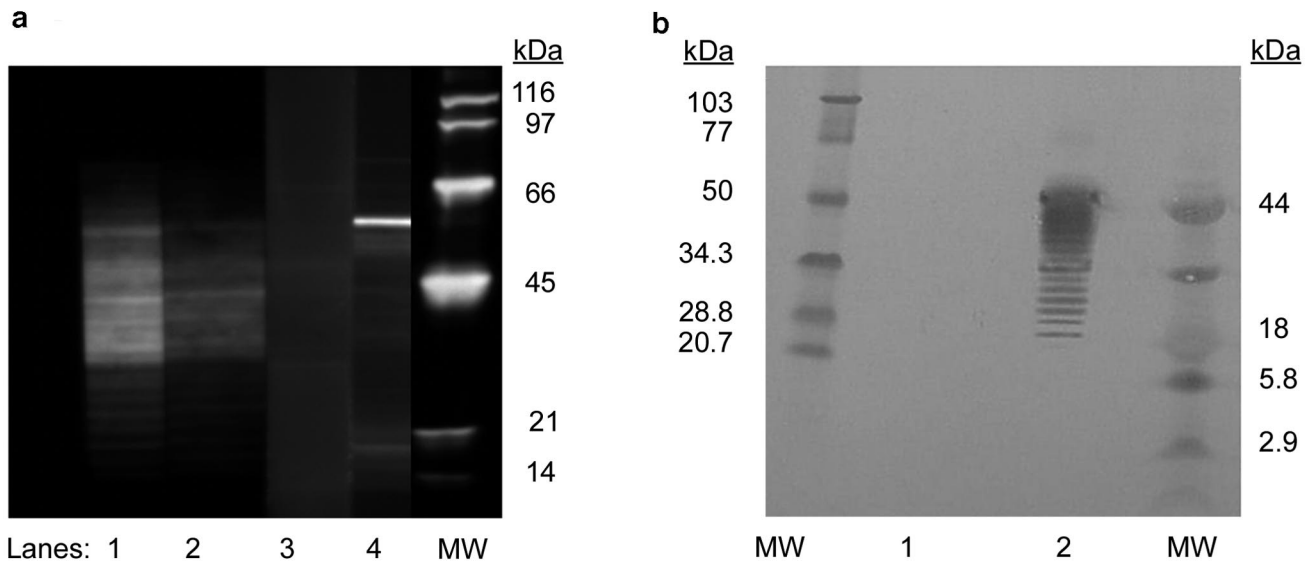
a

b

FIG. 1. (a) Immunoblot analysis of two representative anti-*B. mallei* MAbs. Lane 1, 8G3-1B11 MAb; lane 2, 1G2-1D3; lane 3, preimmune negative mouse serum control (1:1,000); lane 4, positive anti-*B. mallei* mouse serum (1:1,000); MW, molecular mass standards. (b) Immunoblot analysis of proteinase K-treated *B. mallei* and *B. pseudomallei*. MW, high-molecular-mass standards (103 kDa, 77 kDa, 50 kDa, 34.3 kDa, 28.8 kDa, and 20.7 kDa); lane 1, proteinase K-treated *B. pseudomallei*; lane 2, proteinase K-treated *B. mallei*; MW, low-molecular-mass standards (44 kDa, 18 kDa, 5.8 kDa, and 2.9 kDa).

least at the dose of antibodies (1.0 mg/mouse) used in the experiment. However, our results using an in vitro system of *B. mallei* infection of J774 murine macrophages suggest that the number of intracellular pathogens decreased in infected cells cultured in the presence of anti-*B. mallei* MAbs and complement, compared to that in infected cells cultured in the absence of antibodies and complement (unpublished observations). Therefore, it is conceivable that circulating antibodies at sufficient concentrations could have a bacteriostatic or bactericidal effect on intracellular pathogens in vivo. Similar passive protection against i.p. *B. pseudomallei* infection was previ-

ously reported. Polyclonal antisera specific for the polysaccharide of *P. pseudomallei* LPS were shown to passively protect against an i.p. *P. pseudomallei* infection in a diabetic rat model of melioidosis (3). Likewise, murine MAbs specific for *B. pseudomallei* polysaccharide passively protected mice challenged i.p. with *B. pseudomallei* (9). Thus, the presence of circulating antibodies at the initial stages of infection may contribute to protective immunity against glanders and melioidosis, two clinical conditions caused by the phylogenetically closely related *B. mallei* and *B. pseudomallei*, respectively (8). However, as in most diseases caused by intracellular pathogens, in addition to specific humoral immunity, a
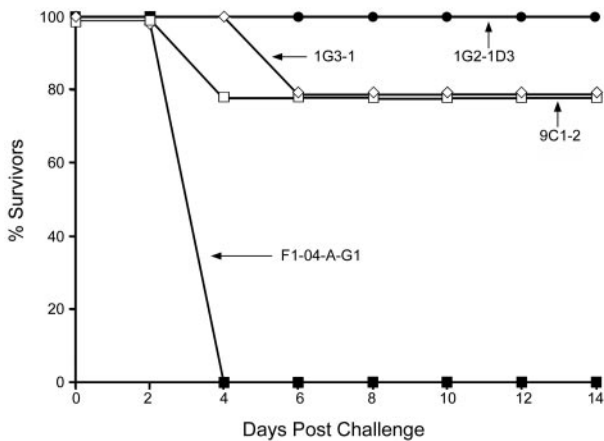

FIG. 2. Passive protection by anti-*B. mallei* MAbs against a lethal aerosol challenge of *B. mallei*. Groups of six mice each were treated i.p. with anti-*B. mallei* MAbs (□, 9C1-2; ●, 1G2-1D3, ◇, 1G3-1) or a negative control anti-F1 MAb (■, F1-04-A-G1) 18 h before an aerosol challenge with 20 LD$_{50}$ (1 LD$_{50}$ = 1,000 CFU) of *B. mallei* strain China 7 (ATCC 23344).
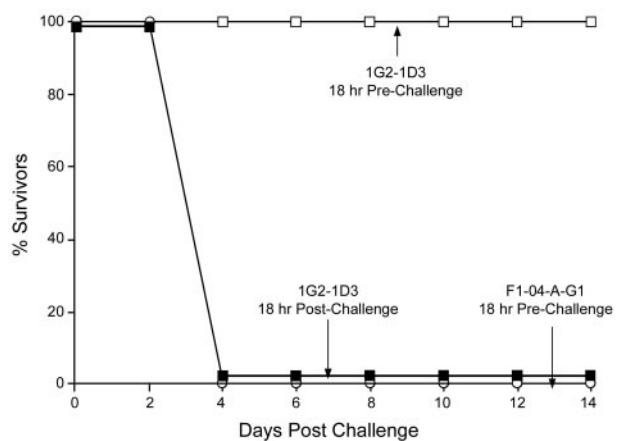

FIG. 3. Lack of passive protection by anti-*B. mallei* MAb 1G2-1D3 given postchallenge. Groups of six mice each were treated i.p. per mouse with 1 mg of either 1G2-1D3 anti-*B. mallei* MAb (□) or F1-04-A-G1 anti-F1 negative control MAb (○) 18 h before an aerosol challenge with 20 LD$_{50}$ of *B. mallei* strain China 7 (ATCC 23344) or challenged and then given per mouse 1 mg of 1G2-1D3 MAb 18 h postchallenge (■).

robust and specific cell-mediated immunity would in all likelihood be necessary for eliminating infected cells and controlling pathogenesis.

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