BACTERIAL PATHOGENICITY

Passive protection against *Burkholderia pseudomallei* infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins

S. M. JONES*, J. F. ELLIS, P. RUSSELL, K. F. GRIFFIN and P. C. F. OYSTON

Microbiology, Dstl, CBS Porton Down, Salisbury, Wiltshire SP4 0JQ, UK and *National Laboratory for Zoonotic Diseases and Special Pathogens, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba R3E 3R2, Canada

***Burkholderia pseudomallei*, the aetiological agent of melioidosis, is endemic in south-east Asia and northern Australia, where it is an important cause of human disease. There is no vaccine available and antibiotic therapy is associated with high relapse rates. A panel of seven monoclonal antibodies (MAbs) that recognise capsular polysaccharide, lipopolysaccharide or proteins was produced and their ability to protect mice passively against experimental melioidosis was evaluated. The MAbs were capable of protecting mice against intra-peritoneal challenge with 10^4 cfu/250 MLD of a virulent strain of *B. pseudomallei* (NCTC 4845), when pooled, and four of the MAbs were individually protective. However, at a higher *B. pseudomallei* challenge level of 10^6 cfu none of the MAbs afforded protection and only the anti-exopolysaccharide MAbs produced a significantly delayed time to death.**

Introduction

Burkholderia pseudomallei, the causative agent of melioidosis, is widely distributed throughout the tropical countries of the world, where it exists as a free-living bacterium in soil and water [1]. The disease is an important cause of human morbidity and mortality in endemic regions; however, the true number of cases is probably much higher than reported because of difficulties in diagnosis [2]. In Australia, melioidosis causes a smaller number of human infections than in countries such as Thailand, but among livestock the disease has important economic and public health implications [3]. Melioidosis can present in many clinical forms, from acute pneumonia or septicaemia to chronic and subclinical forms of the disease. Therapy is complicated by antibiotic resistance in clinical isolates, resulting in frequent relapse of patients [4].

No vaccine licensed for human use currently exists for protection against infection by *B. pseudomallei*. However, a protein and polysaccharide preparation has been used successfully to vaccinate captive marine mammals. This vaccine induced high levels of specific antibodies and resulted in significantly reduced mortality [5]. Avirulent strains of *B. pseudomallei* have also been used to vaccinate animals before respiratory challenge, but with variable results [6, 7].

The lipopolysaccharide (LPS) and flagellin of *B. pseudomallei* have been identified as potential candidates for use as protective immunogens [8, 9]. *B. pseudomallei* has been shown to produce two forms of LPS, termed LPS I and LPS II [10]. Immunoglobulin G (IgG) responses in melioidosis patients to LPS I and II and flagellin have been studied and only the level of antibodies to LPS II was higher in patients surviving infection, indicating their potential immunological importance [11].

It has been suggested that the polysaccharide capsule of *B. pseudomallei* plays a role in environmental protection [12], immune system evasion [13, 14] and attachment to epithelial cells [15]. Capsular polysac-

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Corresponding author: Dr P. C. F. Oyston (e-mail PCOYSTON@dstl.gov.uk).

charide may also allow the bacteria to resist the bactericidal effects of the phagolysosome, enabling the organism to survive within the host [16, 17]. The evidence for this is two-fold. Firstly, a non-capsulate strain of *B. pseudomallei* produced by insertional mutagenesis was markedly less virulent than the parent strain, indicating that the capsule is critical for the virulence of *B. pseudomallei* [18]. Furthermore, another non-capsulate mutant was subsequently shown to be unable to induce a protective immune response in mice, indicating that capsular polysaccharide may be a major protective antigen [19].

After septicaemic melioidosis, serum from survivors contained IgG3 antibodies to LPS O-II polysaccharide [11]. The serum mediated killing of *B. pseudomallei* by polymorphonuclear leucocytes and this effect was enhanced by complement. These results suggest that antibodies specific for LPS O-polysaccharides from *B. pseudomallei* could be protective and this protection might be mediated by enhancement of phagocytosis leading to bacterial clearance.

The present study aimed to achieve passive protection with a range of monoclonal antibodies (MAbs) raised against heat-killed *B. pseudomallei*. The MAbs were specific for LPS, capsular polysaccharide and two proteins.

Materials and methods

Bacterial strain and cultural conditions

B. pseudomallei NCTC 4845 was grown in either nutrient broth or Luria-Bertani medium at 37°C with shaking for 18 h. For viable counts, media were solidified with bacto-agar 15 g/L. The median dose inducing morbidity or mortality (MLD) for this strain is 40 cfu in the BALB/c mouse model (unpublished observations).

Experimental animals

Female BALB/c mice (6–8 weeks old) raised under specific-pathogen-free conditions (Charles River Laboratories, Margate) were used in this study. All animal experimentation adhered strictly to the 1986 Scientific Procedures Act and to the Guidance on the Operation of the Animals (Scientific Procedures) Act (Home Office, UK), as adopted by the ethics committee on animal experimentation within this research establishment.

Preparation of LPS extracts

LPS extracts were prepared according to the method of Chart [20]. *B. pseudomallei* NCTC 4845 was heat-inactivated at 80°C for 2 h in 5 mM phosphate-buffered saline (PBS) pH 7.3. The bacterial cells were harvested by centrifugation and resuspended in SDS-solubilisation buffer (62.5 mM Tris-HCl, pH 6.8, containing

glycerol 10% v/v, 2-mercaptoethanol 5% v/v, SDS 3% w/v and bromophenol blue 0.01% w/v) at a concentration of 1 mg (wet-weight) bacteria/30 µl of SDS-solubilisation buffer. After mixing, the tubes were incubated at 100°C for 10 min. A 30-µl volume of clear, boiled bacterial suspension was added to 30 µl of proteinase K (3.3 mg in 1 ml of SDS-solubilisation buffer) and the tubes were incubated in a water bath at 60°C for a minimum of 1 h. Crude LPS for immunisations was prepared by extensively dialysing the extract followed by lyophilisation.

Immunisation schedule for the production of MAbs

Female BALB/c mice (6 weeks old) were immunised by intraperitoneal injection once every 2 weeks over an 8-week period with heat-inactivated *B. pseudomallei* NCTC 4845. Each dose was equivalent to 2×10^8 cells, determined from viable counts before heat inactivation of the bacterial suspension. A further group of mice was immunised with 50 µg of LPS by the same schedule. The first four injections were given with incomplete Freund's adjuvant. For the final injection, antigen was suspended in PBS and injected intravenously.

Production of MAbs

Three days after the final immunisation the mice were splenectomised and the spleen cells were fused with X63 Ag8.653 myeloma cells as described previously [21]. The culture supernatant fluids of growing clones were screened by ELISA with *B. pseudomallei* NCTC 4845 whole cells as antigen (see below). The resulting hybridomas were cloned by limiting dilution.

ELISA methods

ELISA for the detection of MAbs against *B. pseudomallei* was performed in flat-bottomed Immulon II 96-well plates (Dynex Technologies, Virginia, USA). The plates were coated with heat-inactivated *B. pseudomallei* NCTC 4845 cells (1×10^7 cfu/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The plates were incubated with PBS-M (PBS containing milk powder 1% w/v) for 1–2 h and then washed with PBST (PBS containing Tween-20 0.05% v/v). Hybridoma culture supernatant fluids (100 µl) and culture medium as a negative control were added and the plates were incubated for 1 h. They were then washed with PBST before the addition of horseradish peroxidase-labelled goat anti-mouse immunoglobulin (Harlan SeraLab, Loughborough, UK) diluted 1 in 5000 in PBS-M, and the plates were incubated for 1 h. The plates were washed with PBST before adding 100 µl of substrate solution – 10 mg of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) in 50 ml of buffer (28 ml of 0.1 M Na₂HPO₄ and 22 ml of 50 mM citric acid, pH 4.3) with the addition of 10 µl of H₂O₂.

just before use (OD₄₁₄). The plates were incubated for 15 min at room temperature and the optical density at 414 nm (OD₄₁₄) was measured in a Titertek Multiskan plate reader (ICN Biomedicals, Thame).

Gel electrophoresis and Western blotting

SDS-PAGE was performed according to the method of Laemmli [22] with a 12.5% separating gel (pH 8.45) and a 4.5% stacking gel (pH 8.45); both gels contained SDS 0.1% w/v. Samples were diluted 1 in 2 with sample buffer (SDS 4%, 2-mercaptoethanol 10%, 125 mM Tris-HCl, pH 6.8, glycerol 10% and bromophenol blue 0.004%), boiled at 100°C for 5 min and separated at 100 V for 90 min with Tris-glycine running buffer (50 mM Tris, 380 mM glycine, pH 8.3, containing SDS 0.2% w/v). Polysaccharides were silver stained with the BioRad Silver stain kit and LPS was silver stained according to the method of Chart [23]. Western blotting was performed with Immobilon-P transfer membranes (Millipore, Watford) and a semi-dry blotting system (Biometra, Göttingen, Germany). After transfer, membranes were incubated in bovine serum albumin (BSA) 5% in PBS overnight. MABs were purified by affinity chromatography on protein G-sepharose (Pharmacia, Uppsala, Sweden) and diluted to 1 µg/ml in PBS containing BSA 0.1% plus normal goat serum 1%. Peroxidase-conjugated goat anti-mouse immunoglobulin (Harlan SeraLab) diluted 1 in 2000 in BSA-PBS 0.1% was used for final development. The substrate was 4-chloro-1-naphthol (Sigma).

Passive immunisations

In the first passive immunisation experiment, groups of 10 mice were inoculated intravenously with a cocktail of seven MABs (Table 1). A total of 40 µg of antibody was given to each animal. Of this 40-µg dose, 15 µg was composed of the three MABs to exopolysaccharide, 10 µg of the MAB to LPS and 15 µg of the three MABs to the proteins. The mice were then challenged intraperitoneally with either 10⁶ or 10⁴ cfu of *B. pseudomallei* 4845.

In the second study, groups of five mice were inoculated intravenously with the cocktail of seven MABs described above, 40 µg of a cocktail of the three anti-exopolysaccharide MABs, 40 µg of the anti-LPS

MAB or 40 µg of a cocktail of the three anti-protein MABs.

In the third experiment, groups of six mice were inoculated intravenously with one of the seven MABs. As before, the antibodies were diluted in sterile PBS to a final dose of 40 µg/mouse. The mice were then challenged with 10⁴ or 10⁶ cfu of *B. pseudomallei* 4845. In each experiment the antibodies were administered between 3 and 5 h before challenge and the controls were left untreated. Once challenged, the mice were observed twice daily for either 3 or 5 weeks and mortalities were recorded. At the end of the experiment survivors were killed and observations at post mortem examination were recorded. For all passive immunisation experiments, the significance of the differences in mean times to death was determined by Student's *t* test in Microsoft Excel.

Results

A panel of MABs was produced from animals immunised with heat-inactivated *B. pseudomallei* and, of these, seven were selected for further characterisation (Table 1). Western blotting showed that three of the antibodies recognised a high mol. wt polysaccharide (Fig. 1). Treatment of *B. pseudomallei* cells with sodium periodate inhibited the binding of antibody [19] illustrating that carbohydrate residues are vital for the binding of antibody. Immunofluorescent studies also showed antibody binding in a halo around *B. pseudomallei* cells [19]. These results together support the conclusion that these MABs bound to the capsular

Fig. 1. Western blot of *B. pseudomallei* NCTC 4845 cells with the three anti-EPS MABs: lane 1, 4IIIA11; 2, 3VIE5; 3, 4VA5. All three antibodies recognised high mol. wt polysaccharide material. Pre-stained mol. wt markers were run next to each lane; the highest marker was 120 kDa.

Table 1. MABs used in this study

MAB	Isotype	Target antigen
4VIII AII	IgG2 _{ak}	Exopolysaccharide
3VIE5	IgG2b _λ	Exopolysaccharide
4VA5	IgG1 _κ	Exopolysaccharide
CC6	IgG2 _{ak}	LPS
LF7	IgG3 _λ	Protein
BA2	IgG2 _{ak}	Protein
4VH7	IgG2 _{ak}	Protein

polysaccharide around *B. pseudomallei* [24]. Three further MAb were identified as binding to protein antigens in Western blots (not shown). The function and cellular location of these proteins was not determined. The final MAb was shown to recognise LPS in Western blots (Fig. 2).

The first passive protection study lasted for 3 weeks (Fig. 3) and all mice received a cocktail (40 µg) of the seven MAbs. At the highest challenge dose of 10^6 cfu of *B. pseudomallei* 4845, all the untreated control animals died within the first 5 days. In the equivalent passively immunised group, the last animal succumbed to infection on day 10. Comparing all of the animals in these high challenge groups showed that there was a significant increase in the mean time to death ($p < 0.01$) in the passively immunised group. In the

Fig. 2. Western blot showing the binding of MAb CC6 to LPS from *B. pseudomallei* 4845.

10^4 cfu challenge group, all passively immunised mice survived the entire 3-week observation period. This contrasted with seven deaths in the equivalent control group. Statistical analysis of the mean time to death of these groups demonstrated a highly significant increase in survival ($p < 0.005$). Some of the control animals appeared to develop a more chronic form of infection, which resulted in some survival in the unimmunised control groups, although post mortem revealed that the mice had internal abscesses.

In the second challenge, the duration of the experiment was increased to 5 weeks to ensure that all the untreated controls, including those that initially entered a chronic infection state, would succumb to the infection before the end of the study (Fig. 4). In addition to a group receiving the cocktail of antibodies, three groups were treated with the anti-LPS MAb, a cocktail of protein-specific MAbs or a cocktail of exopolysaccharide-specific MAbs. All groups received 40 µg of antibody per mouse and were challenged with 10^4 or 10^6 cfu *B. pseudomallei* 4845. At the 10^4 cfu challenge dose (Fig. 4a), all the antibody preparations significantly increased the time to death compared with the untreated controls ($p < 0.005$). At the higher challenge dose (Fig. 4b) only the complete antibody cocktail and the exopolysaccharide-specific antibody cocktail gave any significant delay in the time to death compared with the untreated controls (both $p < 0.05$). At this challenge level, all the control animals died by day 3, whereas in the group given the complete cocktail there were survivors until day 18 after challenge, and in the group given anti-exopolysaccharide antibodies two mice survived the 34-day experiment. Mice in both the complete antibody cocktail and anti-exopolysaccharide groups were also significantly more protected than mice in either the anti-LPS or anti-protein groups against the 10^6 cfu challenge. In both the latter treatment groups, all animals died by day 3 at this challenge level.

Fig. 3. Survival in passively immunised and untreated control mice following intra-peritoneal challenge with *B. pseudomallei* 4845. Passively immunised mice were given a cocktail of MAbs recognising exopolysaccharide, LPS and two proteins and challenged with 10^4 cfu (X) or 10^6 cfu (Δ); or naïve (control) mice were challenged with 10^4 cfu (■) or 10^6 cfu (◆). (n = 10, except n = 8 control mice challenged with 10^6 cfu).

Fig. 4. Survival in passively immunised and naive control mice following intra-peritoneal challenge with (a) 10^4 cfu or (b) 10^6 cfu of *B. pseudomallei* 4845. Groups of mice ($n = 5$) were given a cocktail of three MAbs recognising exopolysaccharide (■), a MAb recognising LPS (△), three MAbs recognising proteins (×) or a complete cocktail containing all seven of the MAbs (*). The control groups were untreated.

To determine whether each of the individual antibodies in the cocktails was protective, each MAb was administered independently to groups of six mice, which were then challenged with either 10^4 cfu or 10^6 cfu (Fig. 5). After inoculation with 10^4 cfu of *B. pseudomallei*, all the untreated control mice died by day 4 after challenge. However, in all of the treatment groups, there was significant protection compared with the equivalent untreated group ($p < 0.01$). After 35 days there were survivors in the groups that had received the anti-exopolysaccharide antibodies 3VEI5 or 4VA5, the anti-LPS antibody CC6 or the anti-protein antibody 4VH7. At post mortem examination, all survivors had splenomegaly, with a substantial number exhibiting abscesses on the spleen and liver. In groups that had received a challenge of 10^6 cfu, only mice given 4VA5 or 3VEI5 anti-exopolysaccharide MAbs demonstrated a significantly delayed time to death ($p < 0.05$) compared with the untreated control group. Neither the anti-LPS MAb nor any of the anti-protein MAbs provided significant protection at the 10^6 cfu challenge compared with the untreated control.

Discussion

B. pseudomallei NCTC 4845 causes a rapidly invasive and lethal infection in the BALB/c mouse model when given intraperitoneally. This model of infection appears to be similar to the acute, fulminant, systemic infection seen in many human melioidosis patients. However, melioidosis does have other clinical presentations and these include a chronic abscess-producing infection and a quiescent infection that may take several years to become symptomatic [25].

In this study MAbs to a number of *B. pseudomallei* antigens including the exopolysaccharide, lipopolysaccharide and protein antigens, were produced. As yet, the identities of the protein antigens are unknown and, therefore, it is not possible to speculate on their function. There is a substantial body of data suggesting that capsules are important in the virulence of gram-negative and gram-positive bacteria. In particular, polysaccharide capsules protect bacteria in their interaction with host defence systems, for example, from

Fig. 5. Survival in passively immunised and untreated control mice following intra-peritoneal challenge with (a) 10^4 cfu or (b) 10^6 cfu of *B. pseudomallei* 4845. Groups of mice ($n = 6$) were given one of seven MABs just before challenge: 4III AII (◆), 3VE15 (■), 4VA5 (Δ), CC6 (*), 4VH7 (●), BA2 (+) or LF7 (-).

complement activation and phagocyte-mediated killing [26, 24]. More recently, it has been confirmed that the capsular exopolysaccharide of *B. pseudomallei* is an important virulence factor, as insertional inactivation of the biosynthetic pathway resulted in an acapsular mutant with an LD₅₀ in mice of 3.5×10^5 cfu compared with the wild-type LD₅₀ of <10 cfu [18].

MABs were administered intravenously just before challenge to simulate an infection in individuals with pre-existing high serum titres of specific antibodies, as might be induced by an effective vaccine. The administration of a cocktail of MABs demonstrated a significant protective effect against a challenge dose of 10^4 or 10^6 cfu of *B. pseudomallei*. When the protection afforded by the complete cocktail was compared with that given by the antibodies to specific antigens it appeared that all the specific MABs gave protection equivalent to the complete cocktail at a challenge of 10^4 cfu. However, at a challenge of 10^6 cfu only the complete antibody cocktail and the anti-exopolysaccharide MAB treatment groups demonstrated a significantly delayed time to death.

When the individual MABs were tested against a challenge dose of 10^4 cfu *B. pseudomallei*, the anti-protein MABs provided significant protection compared with the untreated control group. Despite this they appeared to perform less well than the anti-LPS and anti-exopolysaccharide MABs. However, the only MABs to provide any protection against the higher dose of 10^6 cfu *B. pseudomallei* were the anti-exopolysaccharide MABs 3VE15 and 4VA5. The ability of the anti-exopolysaccharide and anti-LPS MABs to protect against *B. pseudomallei* is in agreement with previous observations regarding these antigens and their role in inducing a protective immune response against melioidosis [9, 11, 19]. It seems likely that antibody bound to surface-expressed capsular polysaccharide and LPS enhance phagocytosis by macrophages and neutrophils and may enhance subsequent killing of internalised bacteria. It is also possible that complement fixation and subsequent bacterial killing is another mode of action of these MABs. Certainly, anti-polysaccharide antibodies have been shown to enhance opsonophagocytosis of other gram-negative bacteria [27]. Serological studies suggest that anti-

bodies against LPS II are protective against fatal melioidosis [11] rather than antibodies against LPS I. It is not known which LPS is recognised by MAb CC6 and further work would be required to determine this. However, we have shown that strain 4845 produces a typical *B. pseudomallei* LPS profile (data not shown), and the MAb reacts with this in Western blots.

It is not clear how the antibodies to the protein antigens function. These proteins may be surface-expressed and, therefore, the MAbs against them may act in the same ways as the antibodies against capsular polysaccharide and LPS. However, it is also possible that these are secreted proteins with an essential function, which the antibodies impair or prevent. Further work to identify these proteins is currently taking place and they may be potential components of a sub-unit vaccine for *B. pseudomallei*.

In the second challenge experiment, it was believed that the breakthrough in protection seen in the treated groups towards the end of the challenge might have been due to degradation of the passively transferred antibodies. However, administration of a second dose of MAbs on day 18 did not significantly increase survival in any treatment group (data not shown). This is a result of some significance because it suggests that the limit of protection was not determined by the amount of antibody alone. This would suggest that antibody is only protective in the initial stages of infection and is then less important or ineffective. The most likely explanation for this would be if the bacteria were living intracellularly and that once within a host cell they are able to spread with little or no extracellular phase. It is known that *B. pseudomallei* can invade epithelial cells and grow intracellularly within phagocytes [28, 29]. Thus, the MAbs could act to prevent the colonisation of the intracellular niche, but once the bacteria had established an intracellular infection the presence of high titre, specific antibody may be irrelevant. The immune response induced in mice that survived challenge was not determined and the protection afforded by the MAbs may have been augmented by innate or induced immune mechanisms in the survivors.

A strong cell-mediated cytotoxic response would be required to eliminate infected cells. Previous work has shown that survival in acute infection depends on the production of interferon (IFN)- γ by both NK cells and CD8⁺ T cells. The role of IFN- γ in this case would appear to be to induce both NO- and oxygen-dependent killing of bacteria within infected phagocytes [30, 31]. Unfortunately, passive administration of MAbs does not stimulate this kind of cell-mediated immune response; this would require active immunisation.

The presence of high titre, highly specific antibodies at least to the capsular polysaccharide and LPS (and possibly other components) contributes to the initial

control of infection. However, an optimal vaccine would be required to induce potent specific cell-mediated immune responses that would be able to clear infected cells and prevent *B. pseudomallei* from establishing a chronic infection. How this is to be achieved is currently unknown but the development of a live attenuated mutant of *B. pseudomallei* may induce this type of immune response.

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