Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis


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Burkholderia pseudomallei is the causative agent of melioidosis, which is a major cause of morbidity and mortality in endemic regions. Currently there is no human vaccine against melioidosis. In this study, LPS or capsular polysaccharide was used to immunize BALB/c mice. The different polysaccharide antigens induced antibody responses. Mice vaccinated with LPS developed predominantly IgM and IgG3 responses. Contrastingly, mice vaccinated with capsular polysaccharide developed a predominantly IgG2b response. After immunization, mice were challenged by the intra-peritoneal route and an increased mean time to death was observed compared with unvaccinated controls. Immunization with LPS provided an optimal protective response. Mice challenged by the aerosol route showed a small increase in the mean time to death compared with the unvaccinated controls. The passive transfer of antigen from immunized into naive mice provided protection against a subsequent challenge. This study is the first time antigens protective by active immunization have been identified and suggests that polysaccharides have potential as vaccine candidates against melioidosis.

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis and is a major cause of morbidity and mortality in southeast Asia, particularly Thailand, Vietnam and northern Australia (Chaowagul et al., 1989). Clinically, melioidosis may present as an acute, subacute or chronic infection, which progresses systemically giving rise to the septicemic disease. Untreated septicemia has a mortality rate of between 80 and 90 %, with death occurring within 24–48 h after the initial onset of symptoms (Ashdown, 1992). The current recommendation for antibiotic treatment is high-dose intravenous ceftazidime or a carbapenem typically administered for 10–14 days (Chaowagul, 2000; Dance, 2002). Indeed, early treatment with ceftazidime can decrease mortality by as much as 50 %, although the rate of relapse is still high (White et al., 1989). Currently, there is no vaccine for human use against melioidosis.

A range of surface polysaccharides have been identified in B. pseudomallei, and some of these polysaccharides are also present in Burkholderia mallei. The capsular polysaccharide has been identified as a major virulence determinant of B. pseudomallei strains 1026b and 576 (Reckseidler et al., 2001; Atkins et al., 2002). Capsular material isolated from B. pseudomallei consists of an unbranched homopolymer with the structure -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-. The O-polysaccharide of the LPS consists of an unbranched heteropolymer consisting of (-3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranosyl (Knirel et al., 1992; Perry et al., 1995) and has been reported to play a role in resistance to serum killing and virulence in the diabetic rat model of infection (Woods et al., 1993; DeShazer et al., 1998). Monoclonal antibodies (mAbs) raised against LPS or capsular polysaccharide have been shown to passively protect against B. pseudomallei infection in the BALB/c mouse model (Jones et al., 2002).

A number of polysaccharide vaccines are currently licensed including those to combat Haemophilus influenzae, Neisseria meningitidis and Streptococcus pneumoniae infection (Lee et al., 1991). These produce a T-independent immune response with the production of IgM opsonizing and eliminating the bacteria (Stein, 1992). In order to convert the response to a more favourable T-dependent response polysaccharides have often been conjugated to proteins. This is the case with the H. influenzae type b vaccine (Hib) and the meningococcal type C vaccines (Makela, 2003).

The present study investigates the use of LPS and capsular polysaccharide as subunit vaccine candidates against intra-peritoneal (i.p.) and aerosol challenge in a murine model of melioidosis.

Abbreviations: i.p., intra-peritoneal; MLD, median lethal dose; MPL, monophosphoryl lipid A; MTTD, mean time to death; NCTC, National Collection of Type Cultures; RAS, Ribi Adjuvant system.

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METHODS

Bacterial strain and culture conditions. B. pseudomallei NCTC 4845<sup>T</sup> was used for this study unless otherwise stated. Bacteria were cultured on nutrient agar (bioMérieux) or statically in nutrient broth at 37 °C for 18 h. For enumeration of bacteria, samples were serially diluted in nutrient broth before plating. Brain heart infusion (BHI) plates containing 5% glycerol were used to increase capsular polysaccharide yield. The Advisory Committee on Dangerous Pathogens (ACDP) lists B. pseudomallei as a category III pathogen. All manipulations were carried out in class III microbiological safety cabinets located in designated ACDP containment level III laboratories.

Animals. Female BALB/c mice, 6–8 weeks-old, raised under specific pathogen-free conditions (Charles River Laboratories), were used in this study. Mice were grouped in cages of five with free access to food and water and subjected to a 12 h light/dark cycle. Once challenged the animals were handled under animal containment level III conditions within a half-suit isolator compliant with British Standard BS5726. All investigations involving animals adhered to the requirements of the Animal (Scientific Procedures) Act 1986.

Active immunization and challenge. Groups of ten mice were immunized i.p. with the appropriate antigen on day 0 and boosts were administered on days 14 and 28. Immunization groups were as follows: LPS (25 μg/ml<sup>T</sup>); LPS (25 μg/ml<sup>T</sup>) and a Ribi Adjuvant system (RAS) containing monophosphoryl lipid A (MPL) and TDM emulsion (1:1) (Sigma); capsular polysaccharide (25 μg/ml<sup>T</sup>); capsular polysaccharide (25 μg/ml<sup>T</sup>) plus RAS (1:1). The control mice were not immunized or immunized with RAS adjuvant only according to the schedule above. Mice were tail-bled 4 weeks after the last immunization and either immunized with RAS adjuvant only according to the schedule above. Mice were challenged 7 days later or terminally cardiac punctured for sera. Blood was pooled for each antigen and sera were recovered by centrifugation.

For challenge, groups of mice were given a 0.1 ml suspension i.p. containing 2 × 10<sup>4</sup> c.f.u. B. pseudomallei NCTC 4845<sup>T</sup> or exposed to an aerosol suspension (retained dose 12.5 c.f.u. per mouse) (Russell et al., 1998). We have previously calculated the median lethal dose (MLD) in the BALB/c mouse model to be 40 c.f.u. by the i.p. route and 5 c.f.u. by the aerosol route (unpublished data).

Passive immunization. Groups of five mice were dosed with 0.5 ml of appropriate mAb or polyclonal sera 3 h prior to challenge with 5 × 10<sup>4</sup> c.f.u. B. pseudomallei NCTC 4845<sup>T</sup> per mouse. Mice were dosed with sera taken from mice immunized with LPS or capsular polysaccharide, or with mAb 4V55 or CC6 which recognized capsular polysaccharide or LPS, respectively. Control mice were given naïve sera or PBS. Mice were re-dosed every 3 or 4 days for 10 days, and observed for a further 24 days.

Extraction of capsular polysaccharide. Capsular polysaccharide was isolated from B. pseudomallei NCTC 4845<sup>T</sup> according to the method of Steinmetz et al. (1995). Briefly, the bacteria were grown on BHI plates containing 5% glycerol for 72 h, harvested into PBS and stirred vigorously for 1 h. The solution was centrifuged for 4 h at 20 000 g, the supernatant decanted, heated to 80 °C for 30 min before a further centrifugation at 20 000 g for 30 min. Polysaccharide was precipitated by adding absolute ethanol to a final concentration of 80% (v/v) and incubating at −20 °C for 2 h. The precipitate was collected by centrifugation at 3000 g for 30 min at 4 °C, washed with 80% ethanol for 30 min, pelleted, washed in 96% ethanol and re-pelleted. The precipitate was dissolved in PBS (containing 10 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>, 100 μg RNase A ml<sup>−1</sup> and 100 μg DNase I ml<sup>−1</sup>) and incubated for 2:5 h at 37 °C before heating to 80 °C for 30 min to inactivate the enzymes and centrifuged at 20 000 g for 30 min at 4 °C. A second precipitation was carried out as above with 80% ethanol and after centrifugation the precipitate was dissolved in 2 ml dH<sub>2</sub>O. The suspensions were then lyophilized. The capsular polysaccharide was then affinity-purified using a mAb specific for capsular polysaccharide (3V1E5). The mAb was cross-linked to Protein A-Sepharose CL-4B (Amersham Biosciences) as described by Schneider et al. (1982). The specific affinity matrix was used as a column with a 1 ml bed volume. Capsular polysaccharide (2 ml aliquots) was passed through the column three times and the matrix was washed with 5 ml buffer 1 (0.5 M NaCl, 0.05 M Tris/HCl, pH 8.2, 1 mM EDTA, 0.5% Nonidet P-40) followed by 5 ml buffer 2 (0.15 M NaCl, 0.05 M Tris/HCl, pH 8.2, 0.5% Nonidet P-40, 0.1% SDS) and 2 × 5 ml washes of buffer 3 (0.15 M NaCl, 0.5% sodium deoxycholate). Antigens bound to the matrix were eluted with 3 × 1 ml vols sodium phosphate (0.02 M, pH 12) containing 0.5% sodium deoxycholate. The eluted material was neutralized by the addition of NaH<sub>2</sub>PO<sub>4</sub>.

Extraction of LPS. LPS was extracted from lyophilized B. pseudomallei strain K96243 (provided by S. Songsivilai, Department of Immunology, Mahidol University, Bangkok, Thailand). Bacteria were incubated with lysozyme [20 mg (g bacteria)<sup>−1</sup>] at room temperature for 8 h as previously described by Johnson & Perry (1975) prior to extraction of LPS with hot phenol (Westphal & Jann, 1965). The phenol and water phases were both included in the dialysis step.

Gel electrophoresis, silver staining and immunoblotting. Heat-killed whole-cell samples were treated with proteinase K (Chart, 1994) prior to electrophoresis. Glycine SDS gel electrophoresis was performed using a buffer system described by Laemmli (1970) using a 12.5% separating gel with a 4.5% stacking gel in a Mini-Protean II slab system (Bio-Rad). Gels were silver-stained according to the method of Chart (1994). Immunoblotting was performed by blotting with a semi-dry transfer system and blocked overnight with 5% (w/v) skimmed milk powder and washed 3 × 5 min in 0.1% (w/v) skimmed milk powder. The membranes were probed with mAb 4V55 diluted 1:25 in 0.1% (w/v) skimmed milk powder for 1 h at room temperature. This was followed by 3 × 5 min washes in 0.1% (w/v) skimmed milk powder. An anti-mouse horseradish peroxidase labelled antibody (Amersham Pharmacia Biotech) was diluted 1:1000 in 0.1% (w/v) skimmed milk powder and applied for 1 h. The blot was then washed 3 × 5 min in 0.1% (w/v) skimmed milk powder and twice in PBS. The blots were developed using ECL detection reagents (Amersham), and appropriate equal volumes of reagents 1 and 2 were mixed and applied to the polysaccharide side of the membrane. The membrane was incubated for 1 min and the blots were developed in a darkroom using Kodak Developer and Fixer (Sigma) made as per the manufacturer’s guidelines.

ELISA. ELISAs were performed on sera collected at 4 weeks post-vaccination. Microtitre plate wells were coated with 5 μg per well of either LPS or capsular polysaccharide in PBS and incubated overnight at 4 °C. Wells were blocked with 100 μl 1% (w/v) skimmed milk powder in PBS at 37 °C for 60 min and washed three times in PBS-Tween. Serum samples were double diluted across the plate in skimmed milk powder. To construct a standard curve, more wells were coated with 5 μg anti-Fab antibody ml<sup>−1</sup> and washed as described above. The appropriate isotype standard was double diluted across the plate. Each plate included normal mouse serum as a negative control. The plate was incubated for 1 h at 37 °C and washed three times in PBS-Tween. One hundred microlitres of a 1:5000 dilution of isotype specific goat anti-mouse horseradish peroxidase conjugate (Oxford Biotechnology) was added to each well and the plate was incubated for 1 h at 37 °C. The plate was washed a further six times in PBS-Tween then 100 μl ABTS substrate was added to each well. The plates were incubated at 37 °C for 15 min prior to measuring the OD<sub>414</sub> of the well contents.

Statistical analysis. One-way analysis of variance was the statistical test used where appropriate.
RESULTS AND DISCUSSION

Isolation of LPS and capsular polysaccharide

The homogeneity of the LPS from \textit{B. pseudomallei} has been demonstrated serologically (Pitt \textit{et al.}, 1992; Bryan \textit{et al.}, 1994) and structurally (Perry \textit{et al.}, 1995; Knirel \textit{et al.}, 1992), although atypical LPS banding patterns have been identified (Anuntagool \textit{et al.}, 1998; Perry \textit{et al.}, 1995; Knirel \textit{et al.}, 1992). In-house data suggest that the LPS from \textit{B. pseudomallei} NCTC 4845\textsuperscript{T} and K96243 is similar. This general highly conserved nature suggests that intra-strain protection is applicable. In this study, LPS was extracted from 3 g dry weight \textit{B. pseudomallei} K96243 giving a yield of 9.4 %. The LPS was visualized after SDS-PAGE by silver staining producing a characteristic ladder pattern (Fig. 1a). Capsular polysaccharide was extracted from \textit{B. pseudomallei} NCTC 4845\textsuperscript{T} and appeared as a high molecular mass band in an immunoblot (Fig. 1b).

Immune responses in immunized animals

The LPS or capsular polysaccharide was used to immunize groups of 10 mice three times at 2-weekly intervals. The antibody subclass concentrations in the serum of these mice were determined (Fig. 2). Mice vaccinated with LPS developed IgM and IgG3 responses of $2.2 \times 10^2$ and $1.4 \times 10^3$ ng ml$^{-1}$, respectively. Contrastingly, mice vaccinated with capsular polysaccharide showed a low IgM response of $2 \times 10^2$ ng ml$^{-1}$, a negligible IgG3 response, and predominantly an IgG2b response of $2.4 \times 10^2$ ng ml$^{-1}$. The antibody response to LPS is expected to be an IgG3 response, although when used as an adjuvant LPS can also stimulate IgG2b and IgG2a responses to protein antigens (Kourounakis & Moller, 1984). In septicaemic melioidosis, IgG3 antibodies to LPS are found in the serum of human survivors (Ho \textit{et al.}, 1997; Charuchaimontri \textit{et al.}, 1999). Capsular polysaccharide, however, would be expected to produce more of an IgG3 response (van de Wijgert \textit{et al.}, 1991).

Protection against i.p. challenge

Groups of 10 naïve or immunized mice were challenged with $2 \times 10^4$ c.f.u. (approx. 250 MLDs) \textit{B. pseudomallei} strain NCTC 4845\textsuperscript{T} by the i.p. route and monitored for 35 days (Fig. 3). Mice immunized with either of the polysaccharide antigens showed an increased mean time to death (MTTD). The unvaccinated control mice exhibited a MTTD of 2-6 days, with 100 % mortality occurring by day 11. The highest level of protection was observed in mice that had been immunized with LPS, where 50 % survival was seen at day 35 with a MTTD of 17-6 days. Mice immunized with capsular polysaccharide had a MTTD of 10-5 days and showed 100 % mortality by day 28.

Both antigens behaved differently when co-immunized with RAS. The RAS used in this experiment is a combination of monophosphoryl lipid A (MPL) and trehalose dicorynomycolate. The MPL component is a non-toxic derivative of the lipid A moiety of LPS from \textit{Salmonella minnesota}. Mice immunized with the capsular polysaccharide showed greater survival when co-immunized with RAS, showing a MTTD of 15-1 days and 10 % survival at day 35. The protection observed when LPS was co-immunized with RAS decreased, with a MTTD of 10-2 days and 10 % survival observed at day 35. Control mice receiving only the RAS adjuvant gave a MTTD of 6-8 days and exhibited 100 % mortality at day 29. LPS itself is known to be an effective adjuvant and this may explain why the addition of RAS had no beneficial effect on protection. In the case of the capsular polysaccharide, which lacks a lipid A moiety, co-immunizing with RAS may be necessary to provide optimal protection. Interestingly, RAS affords some protection when given on its own. This phenomenon is not uncommon as MPL and trehalose dicorynomycolate have been shown to provide non-specific protection against viral infection (Masihi \textit{et al.}, 1986).

Active immunization using polysaccharides provides some protection against experimental murine melioidosis by the i.p. route; however, the protection observed is not complete. Possibly, the antigens control the course of infection during the early stages, with the immune response that is stimulated unable to eliminate the bacteria. It is well established that \textit{B. pseudomallei} is capable of invading a number of cell lines (Harley \textit{et al.}, 1998), enabling the bacteria to evade the effects of antibody. To improve the efficiency of a subunit vaccine, the polysaccharide component could be complemented with a protein subunit that will stimulate a protective cell-mediated immune response.
Protection against airborne challenge

Mice immunized with LPS or capsular polysaccharides were challenged by the aerosol route with 12.5 c.f.u. (approx. 2.5 MLDs) \( B. pseudomallei \) strain 4845T. All of the unvaccinated mice, RAS dosed or mice immunized with capsular polysaccharide died by day 4 with a MTTD of 3.1, 3.3 and 3.8 days, respectively. Mice vaccinated with LPS and RAS, LPS, and capsular polysaccharide and RAS died by day 5, with a MTTD of 4.2, 3.9 and 3.4 days, respectively. The lack of protection from this route of challenge may be due to a much more acute infection by the aerosol route giving a different disease pathogenesis and requires further investigation.

Passive immunization

To investigate the role of antibody in protection, mice received PBS, naïve sera, polyclonal sera raised against LPS or capsular polysaccharide, or mAbs to LPS and capsular polysaccharide. Mice were dosed three times at 3–4 day intervals and challenged i.p. with \( 5 \times 10^3 \) c.f.u. \( B. pseudomallei \) strain NCTC 4845T 3 h after the initial dosing. Mice receiving PBS died by day 4 post-challenge. Mice receiving naïve sera died by day 10. Mice receiving either polyclonal antibody or mAb to LPS or capsular polysaccharide survived until day 10. However, 3 days after the antibody dosing ceased, mice showed signs of infection and fatalities occurred. Mice dosed with either mAbs or polyclonal antibodies to LPS showed the greatest protection with a MTTD of 23.7 and 29 days post-challenge, respectively. Passive transfer of antibodies CC6 and 4VA5 has previously been shown to be protective in a murine model against i.p. challenge and it has been
suggested that antibody plays an important role during infection perhaps by enhancing phagocytic killing (Jones et al., 1996). Furthermore, mAbs to an LPS flagellin conjugate have shown passive protection in a diabetic rat model (Brett & Woods, 1996). Interestingly, LPS from non-virulent Ara+ B. pseudomallei isolates is immunologically indistinguishable from LPS from virulent Ara– clinical isolates (Anuntagool et al., 1998). Natural exposure to these Ara+ strains (now reclassified as Burkholderia thailandensis) and the subsequent development of an antibody response without overt infection might protect against melioidosis.

This study is the first time antigens protective by active immunization have been identified and suggests that polysaccharides have some potential as vaccine candidates against B. pseudomallei infection. The protection afforded with these antigens is not complete and a further subunit capable of stimulating a cell-mediated immune response would be required for an ideal vaccine.

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REFERENCES
