

## Specificity and Functional Activity of Anti-*Burkholderia pseudomallei* Polysaccharide Antibodies

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**The lipopolysaccharide (LPS) of *Burkholderia pseudomallei*, the causative agent of melioidosis, consists of two O-antigenic polysaccharides designated O-PS I and O-PS II. In this study, the O-PS specificity and functional activity of a protective polyclonal antiserum and an immunoglobulin M (IgM) monoclonal antibody were determined. The polyclonal antiserum recognized both O-PS I and O-PS II, while the monoclonal antibody was O-PS II specific. Both mediated phagocytic killing of *B. pseudomallei* by polymorphonuclear leukocytes. Patients acutely infected with *B. pseudomallei* also produced antibodies to the two O-PSs, but these antibodies were not produced by asymptomatic individuals from an area of endemicity who were seropositive by an indirect hemagglutination test using sonicated heat-killed whole organisms as antigen. IgM antibodies were detected only in patients with localized infection. IgG antibodies were detected in all acutely infected patients, but there was no significant difference in antibody levels among patients with localized infection, patients who survived septicemic illness, and patients who died from septicemic illness. Further analysis of the IgG response revealed production of IgG1 and IgG2 antibodies by all patient groups, while an IgG3 response was seen only in survivors of septicemic infection. IgG4 was not detectable even when a fivefold-lower serum dilution was used. Patient sera also mediated phagocytic killing by polymorphonuclear leukocytes, and the killing effect was enhanced by complement. These results suggest that antibodies to the LPS O-polysaccharides of *B. pseudomallei* are protective by promoting phagocytic killing. The antibodies develop during human infection and may facilitate clearance of the organisms, as seen in a diabetic rat model of *B. pseudomallei* infection.**

Melioidosis is an infection with the gram-negative organism *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*). It is an important cause of morbidity and mortality in Thailand and other parts of Southeast Asia and northern Australia (8). Clinical disease is associated with a localized or septicemic infection, but asymptomatic infection is also very common. In northeastern Thailand, where a population of seven million people is at risk, septicemic melioidosis accounts for approximately one-fifth of community-acquired septicemias. It is therefore as important an etiological agent as *Staphylococcus aureus* and *Escherichia coli* for this condition (6). Although melioidosis does occur in previously healthy individuals, diabetes mellitus and renal disease appear to be particular risk factors (13). *B. pseudomallei* is intrinsically resistant to penicillin and gentamicin, the usual empirical treatment for suspected septicemia in many parts of the developing world. Even under the best circumstances, prolonged treatment with expensive antibiotics such as ceftazidime or amoxicillin-clavulanate is required for clinical cure (18, 20).

Very little is known about immunity against *B. pseudomallei*. Because it is an intracellular organism of mononuclear phagocytes (17), cell-mediated immune processes are likely to be necessary in overcoming an established infection. However, they do not appear to eradicate the organisms, as relapses can

occur many years after the initial infection (7). Antibodies which are bactericidal or promote phagocytosis may have an important role in the initial clearance of the organism. In this context, polysaccharide antigens would be among the first to be encountered by the host. Antipolysaccharide antibodies have been shown to enhance opsonophagocytosis of other gram-negative organisms, such as *Pseudomonas aeruginosa* (15).

In previous studies, we have demonstrated that passive immunization with antibodies to the lipopolysaccharide (LPS) O-polysaccharide (O-PS) antigens of a serologically typical strain of *B. pseudomallei* (304b) raised the median lethal dose by 4 to 5 orders of magnitude for diabetic rats challenged with a heterologous clinical strain of *B. pseudomallei* (5). The two antibodies which were shown to be efficacious included a polyclonal rabbit antiserum raised against *B. pseudomallei* LPS O-PSs conjugated to tetanus toxoid and a mouse monoclonal immunoglobulin M (IgM) antibody to polysaccharide alone. These antibodies also recognized more than 40 clinical strains of *B. pseudomallei* from Thailand. Subsequent structural analysis of the LPS of strain 304b revealed two S-type LPSs differing in the chemical structure of their O-PS components (14). One O-antigen polysaccharide (O-PS I) is an unbranched high-molecular-weight polymer of 1,3-linked 2-O-acetyl-6-deoxy-β-D-manno-heptopyranose residues. The other LPS O-antigen (O-PS II) is an unbranched polymer of repeating disaccharide units having the structure . . .-3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- . . . in which ca. 33% of the L-6dTalp residues bear 2-O-methyl and 4-O-acetyl substitutions while the other L-6dTalp residues carry only 2-O-acetyl substituents. These two O-antigens are present in approximately equal proportions in the LPS produced by this wild-type strain.

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To further evaluate the potential role of the O-antigen polysaccharides in the development of an anti-*B. pseudomallei* vaccine, we have examined the antigen specificity of the protective antibodies and their functional activity. Human antibody responses to the two O-antigen polysaccharides of *B. pseudomallei* were also studied with particular reference to the IgG subclass of the antibodies, as it is known to be an important determinant of both avidity for multivalent polysaccharide antigens and biological function (1).

#### MATERIALS AND METHODS

**O-PS preparations.** Purified samples of O-PS I and O-PS II from *B. pseudomallei* 304b were prepared as described previously (14). Briefly, LPS from late-logarithmic-growth-phase bacteria was extracted by a modified hot aqueous-phenol method. The lipid A moiety was removed by hydrolysis of the LPS in 5% acetic acid (at 100°C for 2 h). The lyophilized water-soluble product was fractionated by Sephadex G-50 gel filtration column chromatography with 0.05 M pyridinium acetate (pH 4.6) as the mobile phase. The pyridinium acetate was prepared from 4 ml of pyridine (Anachemia Co., Ltd., Montreal, Canada) and 10 ml of glacial acetic acid and was made up to 1 liter with distilled water. Fractions collected across the eluate yielded a homogeneous high-molecular-weight polymer (O-PS I) and a lower-molecular-weight polymer (O-PS II). A heterogeneous fraction containing O-PS I and O-PS II (1:1) and designated O-PS was collected from the intermediate eluted void volume fraction. The O-PS I and O-PS II prepared in this manner were of approximately 95% purity. Pure reference samples of O-PS I and O-PS II were also obtained from the mild-acid hydrolysis of the LPS preparations isolated from a mutant strain of *B. pseudomallei*, SRM 117, and an environmental strain, E264, which were unique in producing smooth-type LPS composed of a single antigenic O-PS (4).

**Anti-PS polyclonal and monoclonal antibodies.** A polyclonal IgG antibody to a *B. pseudomallei* O-PS-tetanus toxoid conjugate was produced in a New Zealand White female rabbit by intravenous immunization as described previously (5). The immunoglobulin fraction was ammonium sulfate precipitated. An IgM monoclonal antibody to O-PS (PS-Pp-W) was prepared from antigens eluted from sodium dodecyl sulfate-polyacrylamide gels of *B. pseudomallei* LPS. The specificity of the antibodies was determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting against proteinase K-treated whole cells of *B. pseudomallei* and a variety of clinically relevant gram-negative bacteria (5).

**Clinical specimens.** Sera were collected from five groups of Thai subjects, including healthy controls from areas of Thailand where clinical melioidosis is not seen ( $n = 50$ ); asymptomatic individuals ( $n = 18$ ) from northeast Thailand, where melioidosis is endemic, whose sera were positive for *B. pseudomallei* antibodies by the indirect hemagglutination test (IHA), a screening test commonly used in Thailand which employs antigens from sonicated *B. pseudomallei* that are adsorbed onto sheep erythrocytes; and patients admitted to hospital in Ubol Ratchatani with either localized ( $n = 22$ ) or septicemic ( $n = 41$ ) *B. pseudomallei* infection. The patients with septicemic illness were further subdivided into those who died ( $n = 20$ ) and those who recovered from the infection following appropriate treatment ( $n = 21$ ). Sera were collected from patients at the time of hospital admission. The controls and patients participated in clinical studies which were approved by the Ministry of Health, Bangkok, Thailand.

**ELISA determination of antibodies against O-PS and O-PS I and II.** An ELISA was used to measure antibodies against O-PS, O-PS I, and O-PS II. Polyvinyl chloride plates (Falcon 3911; Becton Dickinson, Oxnard, Calif.) were coated with the polysaccharides at their optimal concentrations of 1 to 5 µg/ml in carbonate buffer, pH 9.6, at 37°C for 2 h. The polyclonal and monoclonal antibodies were tested at dilutions of 1:100 and 1:5,000, respectively. For the clinical specimens, preliminary titrations (1:20 to 1:1,000) were performed with serum samples from three healthy controls and nine acutely infected patients, three each with high, medium, and low responses, in order to determine which serum dilution best distinguished the levels of antibody to O-PS I and O-PS II of control and patient groups. The results indicated that a serum dilution of 1:100 gave good separation of the median absorbance values and was economical on serum. Furthermore, the low absorbance values for IgG3 and IgG4 measurements were not significantly increased by using a serum dilution of 1:50 or 1:20. Therefore, all serum samples from patients were subsequently tested at a 1:100 dilution.

The plates were incubated with the samples at 37°C for 1 h and then washed. Optimal concentrations of peroxidase-labelled goat anti-mouse IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), goat anti-human IgM and IgG (Miles-Yeda, Ltd., Kiryat Weizman, Rehovot, Israel), goat anti-rabbit IgG (ICN ImmunoBiologicals, Costa Mesa, Calif.), and mouse anti-human IgG1, IgG2, IgG3, and IgG4 (Pharmingen, San Diego, Calif.) were then added for 1 h at 37°C. After washing, the substrate 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] was added. After 30 min at room temperature, the absorbance at 405 nm was read on a spectrophotometer. The mean absorbance of duplicate readings was determined.

To confirm the specificity of the protective antibodies, the rabbit antiserum at a 1:100 dilution and PS-Pp-W at a 1:5,000 dilution were preincubated with either

O-PS I or O-PS II at 0.3 to 16.5 µg/ml at 37°C for 1 h. The adsorbed antibodies were spun down in a microcentrifuge, and their reactivities to O-PS, O-PS I, and O-PS II were determined by ELISA as described above.

**Opsonic killing assay.** *B. pseudomallei* 304b was grown overnight in 20 ml of brain heart infusion broth at 37°C in a shaking incubator. Bacteria were then subcultured at an optical density (at 600 nm) of  $0.075 \pm 0.005$  in 50 ml of brain heart infusion broth for a further 3.5 h at 37°C in order to reach mid-log-growth phase. The bacteria were harvested by centrifugation at  $1,000 \times g$  for 10 min and were washed in 10 ml of sterile normal saline. The washed bacteria were resuspended to an optical density (at 600 nm) of  $0.500 \pm 0.005$  and were further diluted 1:75 in Hanks' balanced salt solution (Gibco BRL, Gaithersburg, Md.) containing 0.1% gelatin (gHBSS).

Polymorphonuclear leukocytes (PMNL) were isolated aseptically from the venous blood of a single nonexposed Canadian donor by using a Polymorphprep solution from Nycomed Pharma (Gibco BRL) according to the manufacturer's instructions. The cells were washed once in normal saline and then resuspended at  $5 \times 10^6$  cells/ml of gHBSS. The purity of the PMNL preparation, as determined by the Diff-Quik stain set (Baxter Healthcare Corp., Miami, Fla.), was greater than 99%, as was cell viability, determined by trypan blue dye exclusion.

The assay for opsonophagocytic activity was performed in sterile polypropylene tissue culture tubes (Falcon 2063; Becton Dickinson) which contained various combinations of 250 µl of PMNL, 50 µl of bacteria, 50 µl of hamster serum (complement source), 50 µl of heat-inactivated test serum, and gHBSS to a total volume of 500 µl. The test sera included the polyclonal rabbit antiserum, the monoclonal antibody PS-Pp-W, and sera from patients with localized ( $n = 6$ ) or septicemic ( $n = 5$ ) infection. The numbers of PMNL and bacteria were calculated in order to arrive at a 1:1 effector-target ratio. The tubes were incubated on a roller mixer at 37°C. At 0, 30, and 120 min, 50 µl of cell suspension was removed into 5 ml of sterile distilled water with 0.1% gelatin. The cells were lysed for 10 min, and 100 µl of the suspension was inoculated on agar plates. Viable colony counts were done after incubation overnight at 37°C, and each was expressed both as the absolute count and as a percentage of the colony count from bacteria plated at 0 min. Preliminary experiments using bacteria suspended in distilled water for 10 min with 0.1% gelatin and treated in the same manner showed that they retained their viability, while the PMNL underwent complete lysis.

To confirm that the opsonophagocytic effect was *B. pseudomallei* specific, the antibodies were adsorbed with *B. pseudomallei* 304b. Fresh bacteria were boiled in phosphate-buffered saline for 30 min and centrifuged, and the supernatant was removed. Fifty microliters of the bacterial pellet was resuspended in 140 µl of serum for 1 h at 37°C. The mixture was spun down in a microcentrifuge, and the adsorbed serum was used in the opsonophagocytosis assay after a sterility check was performed.

**Bactericidal assay.** The bactericidal activity of the protective antibodies and patients' sera in the presence or absence of complement was determined in an assay identical to that described for opsonophagocytosis, except that PMNL were not added to any of the tubes.

**Statistical analysis.** Median absorbance values for the five study groups were compared with the Kruskal-Wallis nonparametric analysis of variance test. Dunn's multiple comparison test was then used to determine the significance of differences in median absorbance between pairs of groups. Only  $P$  values from Dunn's multiple comparison test are reported. A  $P$  value of  $<0.05$  was considered statistically significant.

#### RESULTS

**Antigen specificity of protective antibodies.** The specificities of the polyclonal rabbit antiserum and the monoclonal antibody were determined by using the O-PS preparations as the capture antigen in an ELISA. The polyclonal antibody recognized both O-PS I and O-PS II, while the monoclonal antibody reacted exclusively with O-PS II (Table 1). In order to ensure that the difference in antigen recognition by the two antibodies was not due to poorer adsorption of O-PS I to the polyvinyl plates, adsorption studies were performed in which the antibodies were preincubated with either O-PS I or O-PS II at various concentrations and their reactivities to O-PS were tested. The results indicate that the reactivity of the polyclonal antibody to O-PS could be partially inhibited by either O-PS I or O-PS II, while the reactivity of the monoclonal antibody to O-PS was completely inhibited by preincubation with O-PS II but not with O-PS I, confirming that it was specific for O-PS II.

**Antigen specificity of patient sera.** Sera from acutely infected patients also recognized both O-antigens. Compared to normal controls, a significant IgM response to both O-PS I and O-PS II was detected only in the sera of patients with localized

TABLE 1. Reactivities of *B. pseudomallei*-specific polyclonal and monoclonal antibodies with O-PS, O-PS I, and O-PS II

Antibody	Absorbance (405 nm) <sup>a</sup>		
	O-PS	O-PS I	O-PS II
<b>Polyclonal (1:100)</b>			
Preadsorption	0.780 ± 0.105	0.483 ± 0.069	0.393 ± 0.052
Postadsorption			
O-PS I	0.383 ± 0.032	ND	ND
O-PS II	0.293 ± 0.039	ND	ND
<b>Monoclonal (1:5,000)</b>			
Preadsorption	0.831 ± 0.102	0.021 ± 0.002	1.953 ± 0.139
Postadsorption			
O-PS I	0.780 ± 0.081	0.054 ± 0.009	1.717 ± 0.072
O-PS II	0.002 ± 0.003	0.014 ± 0.002	0.084 ± 0.010

<sup>a</sup> Results are means ± standard deviations of three experiments performed with antibodies before and after adsorption with 3.3 µg of O-PS I or O-PS II/ml. ND, not done.

infection (Fig. 1a) ( $P < 0.01$  and  $0.001$ , respectively). In contrast, an IgG response to O-PS I and O-PS II was seen in all three patient groups (Fig. 1b) ( $P < 0.001$  compared to controls in all six cases) but not in asymptomatic individuals ( $P > 0.05$  in both cases). However, there were no significant differences in median antibody levels among the three groups of patients

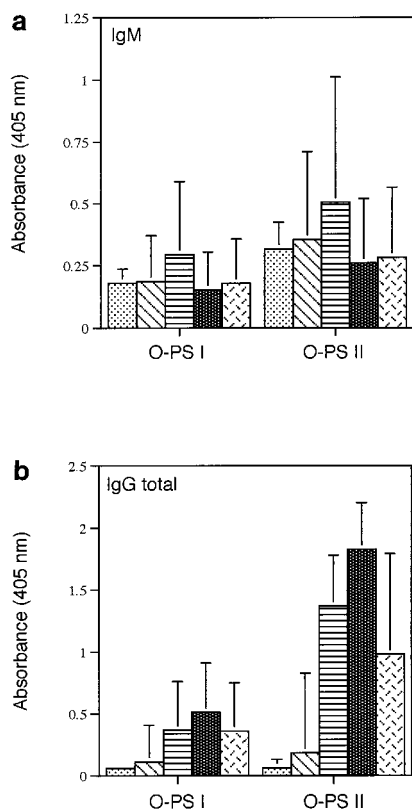


FIG. 1. IgM (a) and IgG (b) anti-O-PS I and anti-O-PS II antibody concentrations in sera of healthy controls (□) ( $n = 50$ ), asymptomatic individuals (▨) ( $n = 18$ ), patients with localized infection (■) ( $n = 22$ ), and patients who survived (▤) ( $n = 21$ ) or died from (▦) ( $n = 20$ ) septicemic *B. pseudomallei* infection. Group median absorbance values plus the upper 95% confidence limits are shown.

(those with localized, nonfatal septicemic, or fatal septicemic illness) ( $P > 0.400$  for all paired comparisons).

The IgG response to O-PS I and O-PS II was further analyzed according to the subclasses (Fig. 2). Compared to healthy controls, asymptomatic individuals had no IgG subclass antibodies to either O-PS I or O-PS II ( $P > 0.05$  in both cases). All three groups of acutely infected patients produced IgG1 antibodies to O-PS I ( $P < 0.05$  in all three cases). Production of IgG2 antibodies to O-PS I was limited to the two groups of patients with septicemic illness ( $P < 0.05$  for survivors and  $P < 0.001$  for fatal cases). The differences in the IgG1 and IgG2 antibody levels among the patient groups were not statistically significant ( $P > 0.05$  for all paired comparisons). IgG3 and IgG4 antibodies to O-PS I were not detected in any patient group, even when the sera were tested at a 1:20 dilution.

Compared to healthy controls, all three groups of infected patients produced IgG1 and IgG2 antibodies in response to O-PS II ( $P < 0.001$  in all three cases). There were no significant differences in antibody levels among the three patient groups ( $P > 0.05$  for all paired comparisons). Interestingly, an IgG3 response to O-PS II was detected only in patients who survived a septicemic infection ( $P < 0.001$  compared to healthy controls). No IgG4 antibodies were detected in any patient group, even when the sera were tested at a 1:20 dilution.

**Bactericidal activity.** To determine if the protective antibodies or patients' sera were directly bactericidal to *B. pseudomallei*, a suspension of the bacteria was incubated in 10% heat-inactivated specific antibody or patient serum in the presence or absence of hamster complement. No bactericidal effect was demonstrated with either the polyclonal or the monoclonal antibody, or with any of the 10 serum samples from patients with localized ( $n = 4$ ) or septicemic ( $n = 6$ ) infection (data not shown).

**Opsonic killing.** *B. pseudomallei* remained viable when incubated for 120 min with PMNL resuspended in HBSS containing 10% heat-inactivated normal rabbit or human serum, in the absence or presence of 10% hamster serum as a common source of complement (Table 2). The addition of the polyclonal antiserum resulted in 31% growth inhibition, which was increased to 87% in the presence of complement. In contrast, the monoclonal antibody PS-Pp-W produced a two- to three-fold increase in mean viable colony counts but was 90% inhibitory when complement was present. The polyclonal antibody had an opsonic titer of 1:100, while PS-Pp-W had a titer of 1:5,000.

The opsonophagocytic activity of the sera of patients with localized and septicemic illness was also studied (Table 2). Two of five serum samples from patients with septicemic illness promoted the killing of *B. pseudomallei* by PMNL in the absence of complement. The killing effect of these two serum samples accounted for the 33% reduction in viable colony counts shown in Table 2 for this group of patients. Ninety percent or higher killing activity was observed for all serum samples tested when complement was added. The serum samples from patients with localized disease were inhibitory only when complement was present. The opsonic titers for the patient sera ranged from 1:10 to 1:100. For the small number of patients studied, there was no direct correlation between antibody level (absorbance value) and the opsonic titer (data not shown). However, the phagocytic activity was *B. pseudomallei* specific, as it could be completely abrogated in all cases by preincubation of the sera with heat-killed whole organisms.

## DISCUSSION

*B. pseudomallei* is widespread in wet soils in northeast Thailand and other areas of Southeast Asia. The infection is most common in rice-farming communities, where the likelihood of infection depends on the degree of exposure to this environmental organism (19). As a result, the risk is very high during the ploughing and planting of rice paddies. Reducing this risk by wearing protective clothing is not a realistic option. Effective immunization would be a major goal in the control of this disease.

We have previously shown that antibodies against the LPS O-polysaccharide of *B. pseudomallei* raised the median lethal dose by 4 to 5 orders of magnitude for diabetic rats challenged with a heterologous clinical strain of *B. pseudomallei* (5). Subsequently, the O-PS was found to consist of two O-chains which are structurally distinct from each other (14). Similar O-chain structures have been demonstrated in the LPS components of several strains of *B. pseudomallei* isolated in Vietnam (12), and homogeneity of the LPS of *B. pseudomallei* has

also been shown serologically and by immunoblotting (16). Collectively, these findings indicate that there may be only one serotype of *B. pseudomallei* and that an effective LPS vaccine against *B. pseudomallei* would be widely applicable. In the present study, the protective monoclonal antibody was shown to be directed towards O-PS II alone. This O-PS would thus appear to be a prime candidate for a conjugate vaccine, especially if it is conjugated to a homologous protein such as *B. pseudomallei* flagellin. Antibodies to *B. pseudomallei* flagellin protein have also been shown to be protective in a diabetic rat model, reducing the median lethal dose by 3 to 4 orders of magnitude (3).

The specificity of the anti-O-PS antibodies in patients acutely infected with *B. pseudomallei* was also examined. A significant IgM response to both the O-chains was detected only in the sera of patients with localized infection, which may reflect the shorter duration of disease in these patients. On the other hand, total concentrations of IgGs to both O-PSs were elevated in all acutely infected patients. However, there was no significant difference in total IgG levels among patients with different outcomes. Surprisingly, asymptomatic individuals who were positive by IHA had no anti-O-PS antibodies. This finding suggests that either their antibodies were directed to other *B. pseudomallei* antigens or the IHA was not as specific for *B. pseudomallei* as was the ELISA using purified O-PS as the capture antigen. Based on the results of the present study, the determination of total IgG to O-PS II by ELISA may prove to be a useful diagnostic test for the detection of an acute *B. pseudomallei* infection.

As the total IgG levels were similar in patients with localized infection and those with nonfatal or fatal septicemic illness, we next determined if the different clinical outcomes were associated with variations in the pattern of IgG subclass response. This relationship has been demonstrated in infections such as *Plasmodium falciparum* malaria, in which immune individuals were shown to mount an IgG1 and IgG3 response during an infection while those who developed clinical disease produced mainly IgG2 (2). The association of IgG1 and IgG3 with a favorable outcome presumably relates to their intrinsic opsonic potential and their complement binding efficiency, leading to enhanced phagocytic clearance of the parasite. In the case of antibodies against polyvalent antigens, such as bacterial polysaccharides, IgG2 is also highly effective in binding complement and is related to the increased density of repeat carbohydrate epitopes on these antigens (1). In this study, the IgG subclass responses to O-PS I were low and hence difficult to compare. However, no differences in the pattern of IgG1 and IgG2 responses to O-PS II were observed among patients with localized, septicemic, or fatal *B. pseudomallei* infection. On the other hand, the IgG3 antibodies to O-PS II produced by patients who survived a septicemic infection may have contributed to the favourable outcome. This issue is currently being addressed in our laboratory by studying serial IgG subclass levels in different patient groups.

Both the polyclonal rabbit antiserum and the monoclonal antibody enhanced phagocytic killing by PMNL, but they did not have any bactericidal activity. As expected, the functional activity of the monoclonal IgM was completely complement dependent. The polyclonal antiserum, which contained O-PS-specific IgG, mediated a modest degree of killing on its own, presumably through interaction with Fc receptors on PMNL. However, the killing effect was significantly enhanced by complement. A similar observation was seen with sera from patients with septicemic illness, where the killing effect was augmented in the presence of complement. The lack of a direct correlation between anti-O-PS antibody level and opsonic titer

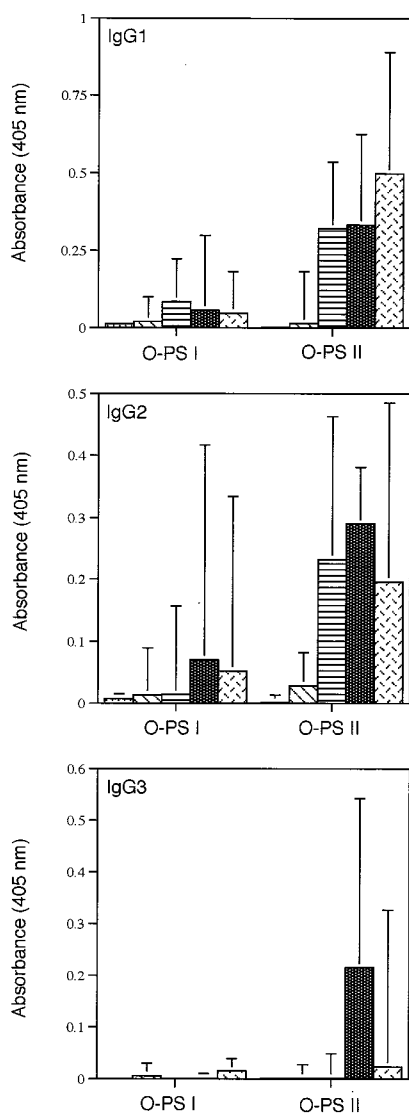


FIG. 2. IgG subclass anti-O-PS I and anti-O-PS II antibody concentrations. Symbols for group medians and 95% confidence limits are as defined for Fig. 1.

TABLE 2. Killing of *B. pseudomallei* incubated with PMNL for 120 min<sup>a</sup>

Serum <sup>b</sup> (n)	Complement	10 <sup>5</sup> CFU <sup>c</sup> after incubation for:		
		0 min	30 min	120 min
Control (4)	–	11.30 ± 5.10	11.75 ± 2.85	11.90 ± 3.25
	+	11.10 ± 4.20	13.25 ± 3.10	13.75 ± 2.80
With Ab Polyclonal (2)	–	14.95 ± 1.40	17.15 ± 2.45	10.10 ± 5.35
	+	11.25 ± 2.55	12.50 ± 1.15	1.50 ± 1.20
Monoclonal (6)	–	11.40 ± 3.20	19.60 ± 1.90	30.00 ± 6.90
	+	11.60 ± 2.45	13.50 ± 3.20	1.25 ± 0.90
From patients With septicemic infection (5)	–	10.50 ± 2.20	13.00 ± 2.77	6.63 ± 3.52
	+	10.12 ± 2.15	4.44 ± 2.25	0.62 ± 0.34
With localized infection (6)	–	10.70 ± 2.42	14.91 ± 2.61	10.54 ± 3.16
	+	10.88 ± 3.56	9.43 ± 1.40	2.29 ± 1.24

<sup>a</sup> All sera were heat inactivated, and 10% hamster serum was used as the source of complement.

<sup>b</sup> Control tubes contained 10% heat-inactivated normal human or rabbit serum. Ab, antibody.

<sup>c</sup> Values are means ± standard deviations of the number of separate experiments indicated (n).

suggests that antibodies to other components of *B. pseudomallei* are also involved in the opsonophagocytic process. Although *B. pseudomallei* has been shown to directly activate complement via the alternative pathway in the absence of antibody (9), this effect was not seen in our experiments using 10% hamster serum as the source of complement.

There is conflicting evidence in the literature regarding the fate of intracellular *B. pseudomallei* in PMNL. Jones et al. (10) and, more recently, Egan and Gordon (9) reported survival of *B. pseudomallei* in PMNL, although a trend for growth inhibition was seen at 60 min in the latter study (9). Even in the present study, we never observed 100% killing, which suggests that a small percentage of bacteria can survive the oxidative and nonoxidative bursts within the phagocyte. The outcome of a phagocytic assay most likely depends on the bacteria-to-PMNL ratio and the duration of incubation used in the experiments. In the studies quoted, the bacteria-to-PMNL ratio varied from 4:1 (9) to 100:1 (10). The 1:1 bacteria-to-PMNL ratio used in the present study would certainly more closely approximate the situation in the bloodstream, where mean colony counts are below 100 CFU/ml. The higher bacteria-to-PMNL ratios used by other investigators may reflect the larger collection of bacteria at tissue sites. The duration of incubation would also appear to be an important determinant of the result of an opsonophagocytic assay in vitro. At 30 min, we also observed survival of *B. pseudomallei*, but a dramatic reduction in mean viable colony counts occurred by 120 min. As we added all the components of the assay simultaneously, the lag period of 30 min may represent the time necessary for optimal opsonization to occur.

More than 80% of children in rice-farming communities in northeast Thailand have acquired antibodies against *B. pseudomallei*, as detected by IHA, by the time they are 5 years old. The vast majority of the adult patients are IHA positive at the time of admission, even if there is a very short history. Since clinical disease resulting from infection with *B. pseudomallei* represents the tip of the iceberg (8), it is possible that antibodies are protective unless there is some breakdown of the immune system, as in patients with diabetes or renal disease. Anti-LPS O-PS antibodies should be effective in the

initial clearance of the organism, but antibodies to other bacterial components or cellular mechanisms may contribute to protective immunity as the infection progresses.

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