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Burkholderia pseudomallei Activates Complement and Is Ingested but Not Killed by Polymorphonuclear Leukocytes

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The mechanism by which *Burkholderia pseudomallei* is resistant to lysis by human serum is unknown but may include interference with complement activation, effective opsonization, or complement-mediated lysis. We investigated the interaction of *B. pseudomallei* with complement in the presence and absence of specific antibody to determine potential mechanisms of serum resistance. We demonstrated rapid activation and consumption of complement by *B. pseudomallei* which, in the absence of specific antibody, occurred predominantly via the alternative pathway. Complement activation was associated with deposition of the opsonically active C3b and iC3b fragments on the bacterial surface. C5b-9, detected on the bacterial surface after opsonic periods of 1 to 60 min, was susceptible to elution by 1 M NaCl, indicating that resistance to complement-mediated lysis may result from deposition of the membrane attack complex in a nonmicrobicidal location. To define the role of opsonins, we investigated the ability of polymorphonuclear leukocytes (PMNL) to phagocytose *B. pseudomallei*. Phagocytosis of bacteria by PMNL, and the observed oxidative response, was significantly increased by opsonization of organisms with complement and/or specific antibody. Despite opsonophagocytosis by PMNL and the production of an oxidative response, no significant bacterial killing was observed.

Burkholderia pseudomallei, the causative agent of melioidosis, produces a spectrum of human disease with protean clinical manifestations, including subclinical infection, acute localized, pulmonary, and septicemic infections, and chronic granulomatous lesions (5, 23). The disease is endemic in Southeast Asia, northern Australia, the Indian subcontinent, Iran, and Central and South America (6). Subclinical or asymptomatic infection is the most common form of melioidosis (3, 23). However, the bacterium may remain latent, causing clinical manifestations of disease up to 26 years following exposure (24). Symptomatic infections are associated with a high mortality rate, a slow response to antimicrobial therapy, and a high rate of relapse despite prolonged treatment (33, 39). Furthermore, recurrent melioidosis usually results from endogenous relapse as demonstrated by the prospective collection and isolation of clonally related strains, identified by ribotyping, from patients with primary and recurrent disease (8). Histopathological examination of specimens from bacteriologically confirmed cases of human melioidosis reveal changes which are neither tissue nor organ specific (29, 40). Such changes range from acute suppurative to chronic granulomatous inflammation, usually associated with tissue necrosis. In addition, numerous intracellular bacteria have been observed in phagocytes (7, 40). These features of melioidosis suggest the presence of unique microbial virulence factors which not only promote tissue invasion and destruction but also permit evasion from normal humoral and cell-mediated immunity. Despite the medical importance of *B. pseudomallei*, remarkably little is known of the pathogenesis of acute or persistent infection.

The complement system is a formidable component of innate immunity, important in first-line defense against invading bacteria. Activation of complement, fixation of C3 fragments to the microbial cell wall, and subsequent assembly of the membrane attack complex are vital for complement-mediated lysis of susceptible gram-negative bacteria. Another important function of complement-mediated host defense is the opsonization of bacteria, which facilitates opsonophagocytosis by professional phagocytes via complement ligand-receptor interactions.

Recent evidence indicates that *B. pseudomallei* is resistant to lysis by human serum (15, 41), although the mechanism is unknown. Mechanisms of complement resistance identified for other serum-resistant gram-negative bacteria include impaired complement activation, ineffective opsonization, and evasion of complement-mediated lytic destruction (13, 16). Impaired C5b-9 assembly, shedding or binding the terminal components at a nonmicrobicidal site may confer protection against complement-mediated lysis (16).

Intracellular localization of *B. pseudomallei* has been documented in vivo (7) and recently in vitro (32). The mechanism of entry into professional phagocytes is unknown, although host-specified endocytosis is most likely (26). Furthermore, phagocytosis of *B. pseudomallei* by human polymorphonuclear leukocytes (PMNL) is reportedly dependent on the presence of thermolabile serum opsonins (34), implicating complement receptor-mediated uptake. The utilization of complement receptor-ligand systems to gain access to the intracellular milieu of phagocytes, thereby avoiding complement attack, has been described for some facultative intracellular bacteria, including mycobacteria (36, 37), *Legionella pneumophila* (28), and *Listeria monocytogenes* (9). The nature of surface-bound C3 degradation products may dictate the nature of interaction between the opsonized bacterium and different complement receptors on human phagocytes (10, 11). Identifying the nature of C3 degradation products fixed to the bacterial surface could therefore assist in determining whether opsonization occurs and if opsonophagocytosis is a potential means by which *B. pseudomallei* gains access to the intracellular compartment.

Following ingestion, susceptible bacteria are killed by the

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oxidative and nonoxidative responses of the phagocyte. Although intracellular persistence of *B. pseudomallei* is documented (21, 32), there are conflicting reports as to its susceptibility to the microbicidal activity of professional phagocytes (32, 34). The susceptibility of intracellular pathogens to phagocytic killing varies with the type of organism, mode of entry into the cell, and state of cell activation and is closely associated with the required virulence mechanisms for intracellular persistence.

The aim of this study was to determine the mechanisms of complement resistance of *B. pseudomallei* and to characterize the nature of opsonic C3 fragments present after opsonization. We also studied the ability of PMNL to phagocytose *B. pseudomallei*, the opsonic requirements for ingestion, stimulation of the oxidative burst during phagocytosis, and the ability of PMNL to kill *B. pseudomallei*.

MATERIALS AND METHODS

Bacteria. *B. pseudomallei*, isolated from the blood of an aboriginal male (strain 2388), was identified by colonial morphology, biochemical reactions, and agglutination with specific antiserum (1, 2). Bacteria were stored in skim milk at −70°C. They were then subcultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated overnight at 37°C with shaking. Bacteria were diluted in fresh broth and grown to log phase before being adjusted to the desired concentration by a spectrophotometric method.

Sera. Human serum was obtained from healthy volunteers, pooled, and stored in aliquots at −70°C (pooled human serum [PHS]). Classical pathway-deficient serum was obtained by treating PHS with 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (Sigma Chemical Co., St. Louis, Mo.) supplemented with 5 mM magnesium (MgEGTA) prior to use. Following this treatment, the classical pathway-deficient serum had no detectable hemolytic activity against sensitized sheep erythrocytes but retained full alternative pathway activity, as assessed by hemolysis of rabbit erythrocytes (27). Serum deficient in both classical and alternative pathway activity was obtained by heat inactivation of PHS at 56°C for 30 min (heat-inactivated human serum [HHS]).

Serum samples from five patients with melioidosis containing antibody to *B. pseudomallei*, as determined by enzyme immunosorbent assay (EIA) and an indirect hemagglutination test (4), were pooled, heat inactivated as described above, and stored at 4°C. Binding of antibody to *B. pseudomallei* was confirmed by indirect immunofluorescence. It was used at a 5 to 10% (vol/vol) concentration as a source of specific antibody.

Serum sensitivity. Bacteria (approximately 5×10^7 log-phase organisms per ml) were incubated in 50% PHS with or without 10% immune serum in a final volume of 1.0 ml at 37°C in a shaking water bath. Viable counts were determined after incubation for 0, 30, 60, and 120 min by standard colony counting techniques using serial dilutions in phosphate-buffered saline (PBS) and overnight culture on nutrient agar. Bactericidal activity was defined as the change in viable counts following incubation under these conditions. Control assays using HHS with and without specific antibody were run in parallel.

Opsonization. Log-phase bacteria were resuspended to the desired concentration in PBS and opsonized in PHS with and without specific antibody in a shaking water bath at 37°C for various periods of time (0 to 60 min). Bacteria were then removed by centrifugation, resuspended in PBS, and used where preopsonized organisms were required. The supernatants were collected and stored at −70°C.

Hemolytic assays. The supernatants obtained by the methods described above after opsonization were used to determine the residual alternative pathway hemolytic activity by an assay using unsensitized rabbit erythrocytes as described previously (27).

Quantitation of bound C3 fragments (C3 EIA). C3 fragments on the surface of opsonized bacteria were quantified by a modified EIA essentially as described previously (12). Briefly, log-phase bacteria opsonized in PHS with and without specific antibody were washed in 0.1% sodium dodecyl sulfate (SDS; Sigma) in PBS and 2×10^8 organisms were plated in triplicate onto 96-well EIA plates (Disposable Products, Adelaide, South Australia, Australia) and adhered by dry desiccation. Nonspecific binding sites were blocked with 200 μl of 0.5% bovine serum albumin (BSA; CSL, Victoria, Australia) in PBS and incubated for 30 min at 37°C. After washing, 100 μl of 1:500 (vol/vol) peroxidase-conjugated goat antiserum to C3 (12) was added for 30 min at room temperature. The plates were rewashed, and 100 μl of substrate (2.2 mM *o*-phenylenediamine [Sigma] and 0.0004% H_2O_2 in 0.1 M citrate buffer) was added. The reaction was stopped after 30 min by the addition of 50 μl of 10% H_2SO_4. PBS–0.05% Tween 80 (Union Carbide, Greenwich, Conn.) was used for all wash steps. The optical density at 490 nm (OD_{490}) was measured with an EIA Autoreader EL310 (Biotek Instruments, Burlington, Vt.). Controls, in which bacteria were incubated with HHS, were assessed in parallel.

Release and detection of C3 fragments. The nature of C3 degradation patterns on *B. pseudomallei* was determined by immunoblotting C3 fragments released by hydroxylamine treatment of opsonized bacteria (12). Briefly, the bacterial pellet obtained after opsonizing approximately 10^{10} organisms in 50% PHS was washed in 1% SDS in PBS to remove noncovalently bound C3. Bacteria were then resuspended in 125 μl of 0.5 M hydroxylamine (Sigma) in 0.5% SDS (pH 10.0) and incubated for 60 min at 37°C to release ester-bound C3 fragments from the bacterial surface. After centrifugation, the supernatant was collected and reduced with 10 mM dithiothreitol (Sigma) in 1% SDS and then alkylated with 22 mM iodoacetamide (Sigma) in 10 mM Tris (Sigma) and 1 mM EDTA (Sigma).

The samples were then analyzed by SDS-polyacrylamide gel electrophoresis in a 7.5% acrylamide gel under reducing conditions. Proteins were transferred to an Immobilon transfer membrane (Millipore Corp., Bedford, Mass.) by Western blotting (immunoblotting). Nonspecific binding sites were blocked in PBS–1% BSA, and the transfer membrane was incubated sequentially at room temperature with 1:250 (vol/vol) goat antiserum to human C3 (Sigma) for 60 min, 1:1,000 (vol/vol) alkaline phosphatase-conjugated donkey antiserum to goat immunoglobulin G (Silenus, Hawthorn, Australia) for 40 min, and finally developed with 0.4 mM nitroblue tetrazolium (Sigma) and 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1 M Tris–0.1 M NaCl–5 mM $MgCl_2$ (pH 9.5).

Binding of C5b-9 to *B. pseudomallei*. The formation of C5b-9 on the surface of opsonized *B. pseudomallei* was examined by use of a modified EIA (25). Washed, preopsonized log-phase bacteria were plated in triplicate onto 96-well EIA plates at 2×10^6 per well and adhered by dry desiccation. Nonspecific binding sites were blocked as for the C3 EIA outlined above. Plates were incubated sequentially at room temperature with 100 μl of 1:80 (vol/vol) rabbit antiserum to C5b-9 (Calbiochem, La Jolla, Calif.) for 30 min, 100 μl of 1:1,000 (vol/vol) horseradish peroxidase-conjugated sheep antiserum to rabbit immunoglobulin (Silenus) for 30 min, and finally 100 μl of substrate (as for C3 EIA). The reaction was stopped with 50 μl of 10% H_2SO_4, and the color reaction was detected at an OD_{490} as described above. Two sets of controls, also performed in triplicate, were included for each experiment. First, bacteria were incubated in HHS and assessed in parallel in the C5b-9 assay to determine nonspecific binding. Second, all samples were duplicated and assayed in parallel for C3 deposition.

In elution experiments, detection of C5b-9 was determined by EIA as outlined, but *B. pseudomallei* was treated for 30 min at 37°C with 1 M NaCl in Hanks balanced salt solution (HBSS; Gibco, Grand Island, N.Y.) with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (20) before dry desiccation to the solid phase.

Human PMNL. PMNL were purified from heparinized venous blood of healthy volunteers by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation on LSM (ICN Biomedicals, Irvine, Kans.). PMNL were washed in Ca^{2+}- and Mg^{2+}-free HBSS with 0.1% gelatin (GHBSS) and finally resuspended in GHBSS with Ca^{2+} and Mg^{2+} at a 10^7/ml. Purity and viability, assessed by trypan blue exclusion, were greater than 95%.

Quantitative radiolabelled phagocytic assay. Phagocytosis of *B. pseudomallei* by PMNL was determined by a radiolabelled phagocytic assay essentially as described previously (38). Briefly, approximately 5×10^6 [^{3}H]adenine-labelled bacteria, 1×10^6 PMNL, and 20% PHS with or without 10% immune serum were incubated in a final volume of 500 μl for 30 min at 37°C in a shaking incubator. Samples in which PHS was replaced with HHS or PBS were also included. For each variable, identical reactions were set up in four polypropylene scintillation vials (Biovials; Beckman, Chicago, Ill.). Following phagocytosis, 3 ml of ACS 11 scintillant (Amersham, Arlington Heights, Ill.) was added to two of the tubes, thus representing cell- and non-cell-associated bacteria. To separate the cell-associated from non-cell-associated bacteria, 3 ml of ice-cold PBS was added to the remaining two tubes and three differential centrifugation steps ($160 \times g$ for 7 min at 4°C) were performed before the final addition of 3 ml of ACS 11 scintillant. All tubes were counted in a Beckman LS 3801 scintillation counter. The percentage of the bacterial population phagocytosed was calculated by the following equation: % uptake = [mean cpm following differential centrifugation (cell associated)/mean cpm in noncentrifuged tubes (cell and non-cell associated)] × 100, where cpm is counts per minute.

Transmission electron microscopy. Samples of PMNL incubated with *B. pseudomallei* in the presence of one or both opsonins (50% PHS and 10% immune serum) for 60 min were fixed with 0.25% glutaraldehyde and osmium and embedded. Sections were stained with 0.5% uranyl acetate and lead citrate. Microscopy was performed with a Phillips CM10 electron microscope operated at 80 kV.

Chemiluminescence assay. Approximately 5×10^5 log-phase organisms were preopsonized in 20% PHS and/or 10% immune serum, washed, and then added to 5×10^5 PMNL in dark-adapted polypropylene scintillation vials (Beckman) with 2 μM luminol to a final volume of 500 μl. Controls, in which organisms were incubated in HHS or PBS prior to addition to the cells, were run in parallel. Chemiluminescence was measured over time with a Beckman LS 3801 scintillation counter set in the out-of-coincidence mode.

Killing of *B. pseudomallei* by PMNL. *B. pseudomallei* and PMNL, at a bacterium-to-cell ratio of approximately 4:1, were incubated shaking at 37°C with 20% PHS with or without 10% immune serum in a final volume of 500 μl. At 60 min, aliquots from the reaction mix were diluted in distilled water and vigorously vortexed to disrupt cells. Viable colony counts were determined following overnight culture on nutrient agar at 37°C. Assays using bacteria incubated in HHS negative for specific antibody were conducted in parallel.

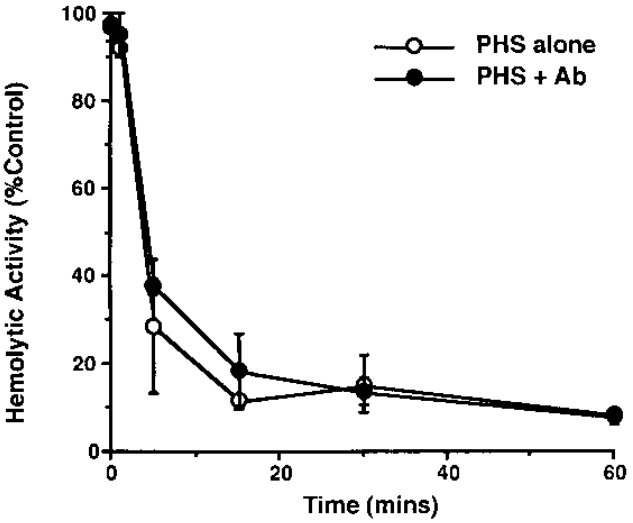

FIG. 1. Complement activation (measured by residual hemolysis of rabbit erythrocytes) by *B. pseudomallei* incubated for 0 to 60 min in 50% PHS with and without 5% immune serum (Ab). Results are expressed relative to the hemolytic activity of control serum from three separate experiments.

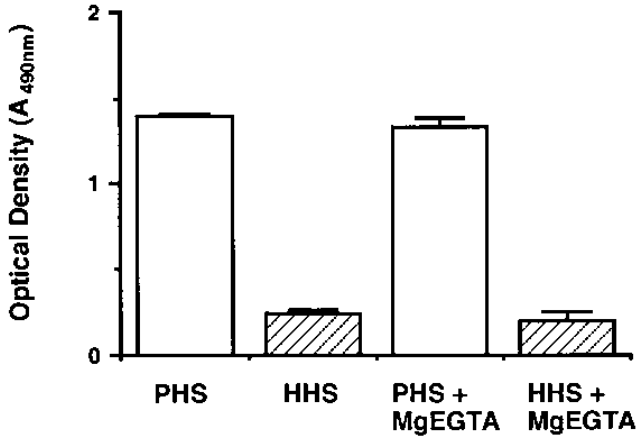

FIG. 2. Alternative pathway activation by *B. pseudomallei* determined by a quantitative enzyme immunoassay detecting surface-bound C3 as measured by optical density (A_{490}). Prior to detection, bacteria were opsonized in PHS or in PHS treated with MgEGTA, in which only the alternative pathway of complement is functional. Shaded areas represent controls for each group in which the respective sera were heat inactivated (HHS) prior to treatment. Results represent the means ± standard errors from three separate experiments, each performed in triplicate.

Statistical analysis. Data from C5b-9 elution experiments were examined by Student's *t* test. All other data were examined by analysis of variance followed by a Newman-Keuls test where appropriate, by using STATPAK 4.12 (NorthWest Analytical Inc., Portland, Oreg.).

RESULTS

Serum resistance. Preliminary experiments confirmed that *B. pseudomallei* is resistant to serum bactericidal activity (15). There was no reduction in viable count during a 2-h incubation, even in the presence of both complement (50% PHS) and specific antibody (data not shown).

Complement activation. The kinetics of complement activation was determined by measuring residual hemolytic activity for rabbit erythrocytes of serum after opsonization of bacteria in 50% PHS for 0 to 60 min in both the presence and absence of 5% immune serum (Fig. 1). There was minimal (<8%) hemolytic activity remaining in serum after incubation with *B. pseudomallei*. The loss of activity occurred dramatically, with a reduction of greater than 50% within 5 min, indicating rapid complement activation. These losses occurred equally in the presence and absence of antibody.

Quantitation of covalent C3 deposition. To confirm that the alternative pathway is the predominant mode of complement activation by *B. pseudomallei*, and that this is followed by complement deposition, a quantitative EIA was performed to detect surface-bound C3 fragments. Log-phase bacteria were preopsonized in PHS or MgEGTA-treated serum. Under opsonic conditions in which only the alternative pathway was functional (MgEGTA-treated serum), the amount of C3 deposited was not significantly different from that obtained when both pathways were potentially functional (Fig. 2).

Kinetic studies in which *B. pseudomallei* was opsonized in 50% PHS with and without specific antibody for various periods of time (0 to 60 min) are shown in Fig. 3a. Greater than 80% of C3 deposition occurred within 2 to 5 min and reached maximal values at 15 min. Increasing the duration of opsonization beyond this time did not increase the amount of C3 deposited nor did the presence of specific antibody. Figure 3b shows the effect of serum concentration on C3 deposition after opsonization of *B. pseudomallei* for 60 min. Despite prolonged incubation, maximal C3 deposition could not be achieved for bacteria opsonized in 10 to 30% PHS. Significantly greater C3 deposition occurred at 50% PHS than at the lowest concentration tested ($P < 0.05$). At each concentration of PHS, the amounts of C3 deposited in the presence and absence of specific antibody were not significantly different.

The role of specific antibody on C3 deposition was then examined in further experiments that were not performed under conditions of relative complement insufficiency or bacterial excess. C3 deposition was determined by C3 EIA as described above, with two exceptions: (i) bacteria were preopsonized in 75% PHS with and without antibody, after which the residual hemolytic activity of serum was 28.6% ± 5.6% (mean ± standard deviation), and (ii) the initial bacterial inoculum was decreased to 1/10th that of the original experiments. In these conditions in which complement consumption was incomplete, the addition of specific antibody to PHS increased C3 deposition by 39.2% ± 2.4% compared with that with PHS alone.

Surface-bound C3 degradation patterns. The nature of the C3 fragments deposited on *B. pseudomallei* during opsonization in 50% PHS for 0 to 60 min was investigated by immunoblotting released fragments after hydroxylamine treatment, which releases ester-bound C3 fragments from the activating surface (22). Deposition of C3 fragments occurred rapidly and could be detected at opsonic intervals as short as 1 min (Fig. 4). Deposition of C3b occurred initially, with cleavage to iC3b detectable at 5 min, as evidenced by the appearance of the 67-kDa α′ chain. At the longer periods of opsonization examined, C3b, iC3b, and other lower-molecular-weight fragments, representing further degradation products, were seen.

Levels of C3 deposition on the surface of glutaraldehyde-fixed and unfixed *B. pseudomallei* as determined by EIA were comparable. Since glutaraldehyde derivatizes amino groups on the bacterial surface, thereby preventing the formation of amide bonds, these results suggest that the majority of deposited C3 is ester bound.

Binding of C5b-9 to *B. pseudomallei*. The formation of the membrane attack complex (MAC) on the surface of the opsonized *B. pseudomallei* was investigated by use of a modified EIA with rabbit antiserum reactive against only the assembled

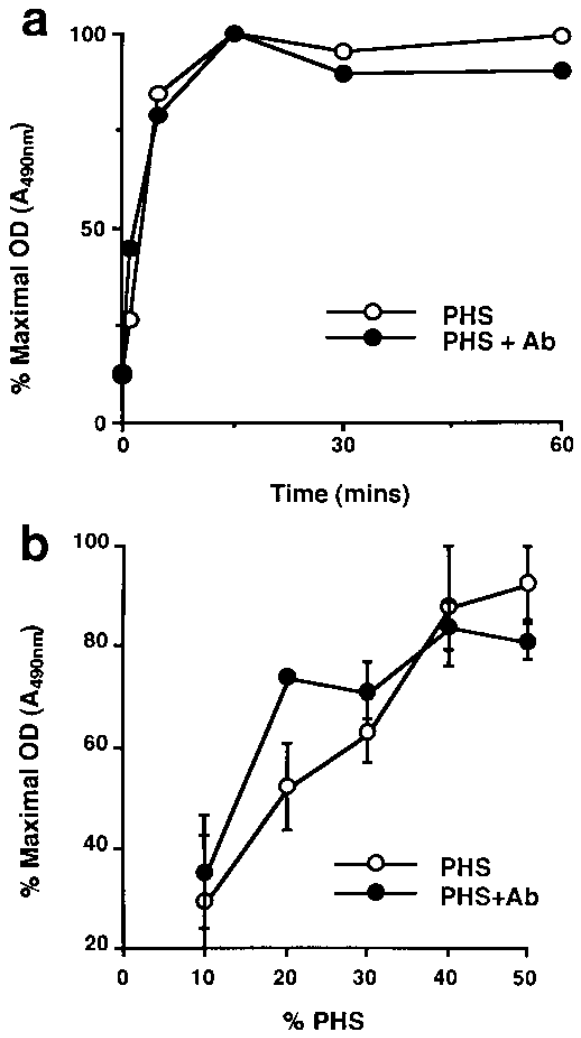

FIG. 3. (a) Representative experiment demonstrating the kinetics of covalent C3 deposition on *B. pseudomallei* opsonized in 50% PHS with and without 5% immune serum (Ab) as determined by EIA. (b) Effect of serum concentration on covalent C3 deposition on *B. pseudomallei* after opsonization in PHS for 60 min in the presence and absence of Ab. Results, expressed as a percentage of maximal optical density (A_{490}), represent means ± standard errors from two experiments, each performed in triplicate.

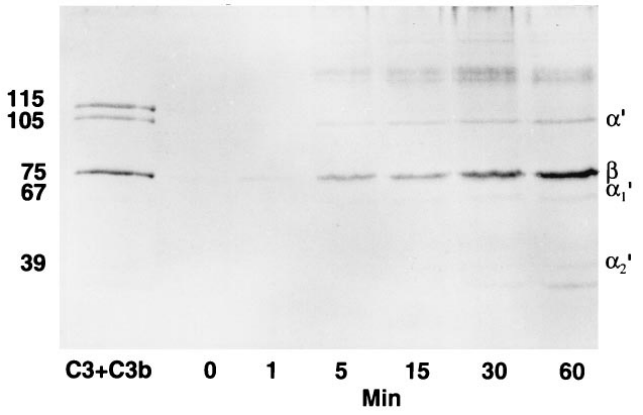

FIG. 4. Immunoblot demonstrating the released ester-bound C3 fragments present on the surface of *B. pseudomallei* after opsonization in 50% PHS for 0 to 60 min. Lanes: 1, bands from a mixture of purified C3 and C3b; 2 to 7, released fragments after opsonization periods of 0, 1, 5, 15, 30, and 60 min, respectively. Sizes are given on the left in kilodaltons. The presence of a 115-kDa chain indicates the α chain of C3, and the 105-kDa chain indicates the α′ chain of C3b. The 67-kDa (α_1' chain) and 39-kDa (α_2' chain) fragments indicate iC3b, and the β chain, common to C3b and iC3b, is represented by the 75-kDa band.

C5b-9 complex. Kinetic studies, in which bacteria were opsonized in 50% serum for 0 to 60 min before application to the solid phase, demonstrated rapid formation of C5b-9 on the bacterial surface similar to that observed for C3 (Fig. 5a), reaching near-maximal levels at 15 min. Conversely, bacteria opsonized for 60 min required at least 30% serum for approximating maximal C3 deposition, indicating that increased duration of opsonization could not compensate for lower serum concentrations (Fig. 5b).

The susceptibility of surface-bound C5b-9 to elution by 1 M NaCl was examined to elucidate the possible site of MAC formation, since association of the complex with the bacterial surface by salt-susceptible bonds suggests that it is not attached by strong hydrophobic interactions with the outer cell membrane (20). The treatment did not affect bacterial viability nor the ability to bind to the solid phase (results not shown). Approximately one-third of the C5b-9 present on bacteria after incubation with 50% PHS was eluted by 1 M NaCl (Table 1). For *B. pseudomallei* preopsonized in PHS and immune serum, 27% of C5b-9 was eluted. Bound C3, which was detected in parallel, was not significantly diminished by incubation in 1 M NaCl.

Phagocytosis of *B. pseudomallei* by PMNL. As determined by a radiolabelled phagocytic assay, the proportions of bacteria phagocytosed by PMNL in the presence of complement and specific antibody or either opsonin alone were 60 and 45%, respectively (Fig. 6). The presence of complement and/or specific antibody significantly increased phagocytosis compared with that of control bacteria incubated with HHS or PBS ($P < 0.01$). Since this method may detect both internalized and surface-associated bacteria, transmission electron microscopy was performed to visualize the site of bacteria. When incubated with *B. pseudomallei* in the presence of PHS and/or immune sera, virtually all PMNL, as examined in numerous fields, were observed to have large numbers of internalized bacteria, indicating that ingestion had occurred. Figure 7 shows the typical appearance from a representative experiment. For unopsonized organisms, only occasional PMNL, containing relatively few bacteria, were observed. For opsonized *B. pseudomallei*, no cell surface-associated bacteria were observed, with the exception of a very few organisms surrounded by pseudopodia typical of conventional phagocytosis.

Chemiluminescence during phagocytosis. The luminol-enhanced chemiluminescence response of PMNL incubated with opsonized bacteria is shown in Fig. 8. A brisk maximal response was observed after the addition of *B. pseudomallei* opsonized with both complement and specific antibody. Lesser responses were observed following the addition of bacteria preincubated with either opsonin alone, but all exceeded that of the control group in which PMNL were incubated with unopsonized bacteria.

Killing of *B. pseudomallei* by PMNL. Having demonstrated that PMNL ingest *B. pseudomallei* relatively efficiently and that an oxidative burst is generated during phagocytosis, we next examined the ability of PMNL to kill this organism. Although there was a trend towards inhibition of growth of *B. pseudomallei* in the presence of opsonins, there was no significant killing of *B. pseudomallei* opsonized with either complement and/or specific antibody over a 60-min period (Table 2).

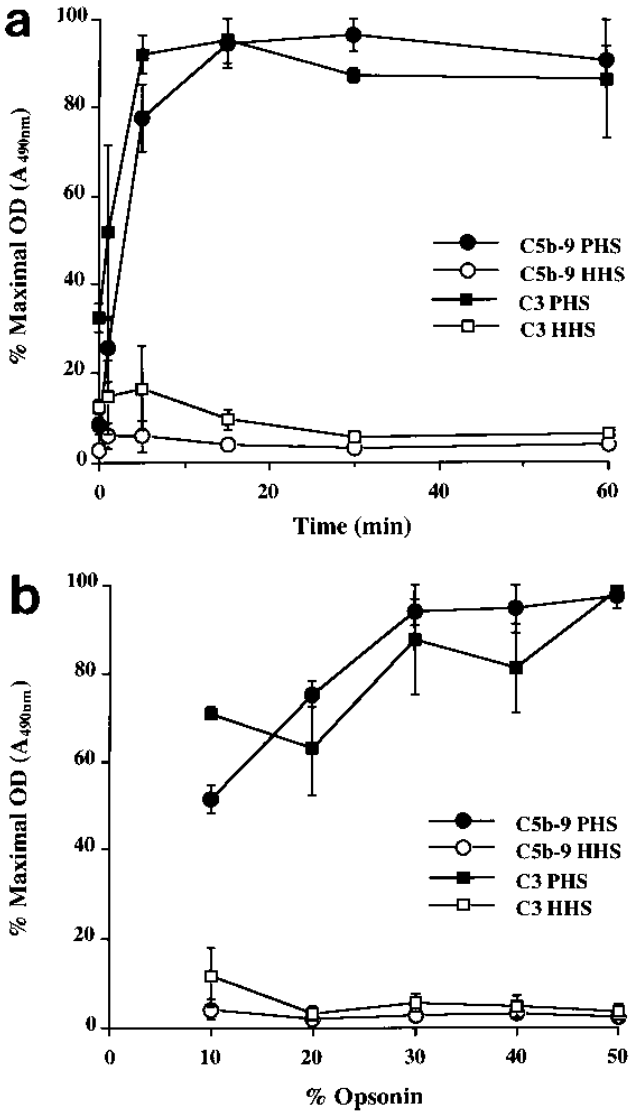

FIG. 5. (a) Kinetics of C5b-9 deposition on the surface of *B. pseudomallei*. Bacteria were opsonized in 50% PHS for 0 to 60 min before dry desiccation to the solid phase and detection of C5b-9 and C3 by enzyme immunoassay as described in Materials and Methods. (b) Effect of concentration of opsonizing serum on C5b-9 deposition. *B. pseudomallei* bacteria were opsonized for 60 min in 10 to 50% PHS before detection as described for panel a. Experiments in which *B. pseudomallei* were incubated in serum heated to deplete complement activity (HHS) were conducted in parallel. Results are from three separate experiments, each performed in triplicate.

DISCUSSION

Recent evidence indicates that *B. pseudomallei* is resistant to serum bactericidal activity (15, 41). We have confirmed and extended this observation to those organisms opsonized with complement and/or specific antibody. Of central importance is whether serum resistance represents a failure of complement activation or subversion of the complement cascade at a later step (13, 16). In this report, we have demonstrated that *B. pseudomallei* is a potent activator of complement. Activation occurred very rapidly, with the majority of serum hemolytic activity lost within 5 min, and was not altered by the presence of specific antibody. Since gram-negative bacteria can activate complement via both pathways in the absence of antibody (17), we used classical pathway-deficient sera to compare, in a quantitative manner, the relative amounts of activation and subsequent C3 deposition that occurred when one or both pathways were functional. The results indicate that in the absence of antibody, the alternative pathway was the predominant mode of activation. The mechanism of complement activation by *B. pseudomallei* was not explored but is likely to include lipopolysaccharide, which is the prototypic activator of the alternative pathway by gram-negative organisms in the absence of antibody (17).

Having demonstrated complement activation, we next examined the amount of C3 fixed to the surface of *B. pseudomallei* and the nature of its degradation products. Covalently bound C3 fragments were quantified by an EIA; C3 deposition was confirmed and occurred very rapidly at high serum concentrations. In contrast, even after prolonged opsonization, serum concentrations of 10 to 30% did not result in near-maximal C3 deposition. Specific antibody did not significantly augment C3 deposition in these experimental conditions, in contrast to those in which complete complement consumption had not occurred. These results further illustrate the importance of high serum concentrations for optimal C3 deposition and may

TABLE 1. Elution of C5b-9 from the surface of *B. pseudomallei* by 1 M NaCl

Opsonin	C5b-9		C3	
	% Elution[a]	*P*[b]	% Elution	*P*
PHS	35.9 ± 7.2	<0.05	13.0 ± 4.8	NS[c]
PHS + Ab[d]	27.4 ± 8.3	<0.05	−8.6 ± 16.1[e]	NS

[a] Data represent the mean percent elution in 1 M NaCl (relative to elution in buffer alone) ± standard error of four experiments, each performed in triplicate.
[b] *P* calculated by Student's *t* test.
[c] NS, not significant.
[d] Ab, 10% immune serum.
[e] Negative result denotes percent increase observed.

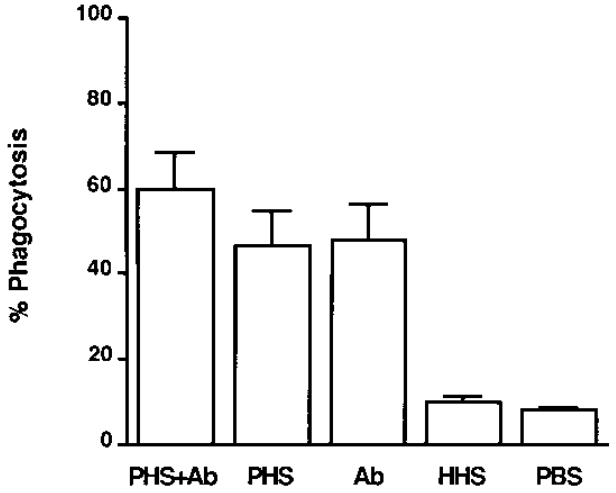

FIG. 6. Percentage phagocytosis by PMNL of *B. pseudomallei*. [^{3}H]adenine-labelled bacteria were preopsonized in 50% PHS with and without 10% immune serum (Ab) or Ab alone before addition to PMNL. Cells were treated with differential centrifugation, as described in Materials and Methods, before detection of cell-associated bacteria by a radiolabelled phagocytic assay. Bacterium/cell ratio, 5:1. The percent phagocytosis by PMNL of control bacteria incubated in HHS or PBS was determined in parallel. The data represent the means ± standard errors from three separate experiments, each performed in duplicate.

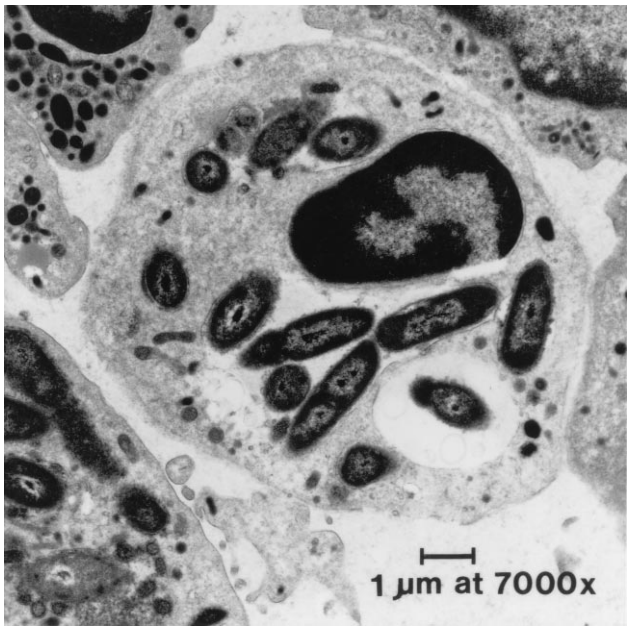

FIG. 7. Representative transmission electron microscopy of human polymorphonuclear leukocytes following incubation with *B. pseudomallei* in the presence of human serum and 10% immune serum demonstrating ingestion and internalization of bacteria.

have significance for opsonization at extravascular sites of infection (12), where latency is often established.

The nature of C3 fragments deposited onto the surface of *B. pseudomallei* will influence the nature of the ligand-receptor interaction occurring between bacterium and complement receptors on phagocytic cells (10, 11). By use of a hydroxylamine release assay, only covalent ester-bound fragments could be directly visualized by immunoblotting. Deposition of C3b occurred virtually instantaneously, followed rapidly by cleavage

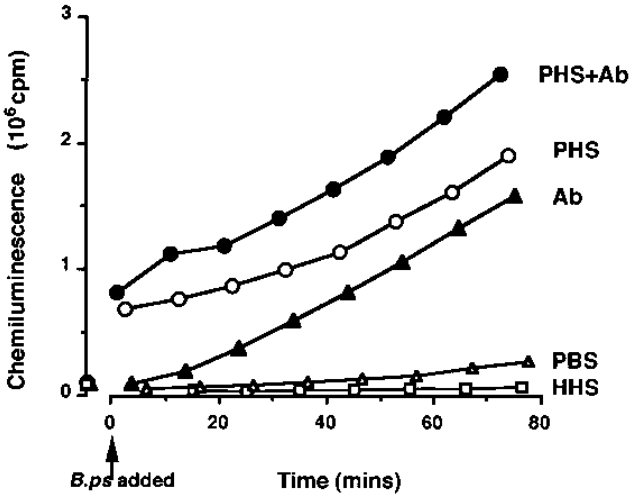

FIG. 8. Representative experiment demonstrating the chemiluminescence response by human PMNL to the presence of *B. pseudomallei*. Bacteria were opsonized in PHS with and without 10% immune serum (Ab) or Ab alone. Background counts were determined before addition of opsonized *B. pseudomallei* (B.ps) to cells in the presence of luminol, after which chemiluminescence was measured over time. Control bacteria, incubated with HHS and PBS, were assayed in parallel. Similar results were observed in three repeat experiments.

TABLE 2. Survival of *B. pseudomallei* incubated with PMNL for 60 min

Opsonin[a]	Colony count (10^7/ml)[b]	
	0 min	60 min
PHS	3.61 ± 1.32	2.68 ± 1.21
PHS + Ab	2.90 ± 1.31	3.01 ± 1.09
Ab	2.27 ± 0.18	3.48 ± 0.67
HHS	3.69 ± 1.66	8.53 ± 4.42

[a] Ab, 10% immune serum.
[b] Values are means ± standard errors of four separate experiments.

to iC3b. These fragments persisted during opsonic periods of up to 60 min. With time, other lower-molecular-weight fragments were detectable, indicating the presence of C3d. Additional higher-molecular-weight fragments containing C3 were also detected. Since SDS, which is used in this assay, may disrupt cellular membranes and cause damage or lysis of *B. pseudomallei*, small membrane fragments with amide-bound C3 may have been responsible for this appearance. To address this, the release assay and all washes were repeated without SDS prior to immunoblotting, and similar degradation patterns were observed (results not shown). Hence, these bands may represent multimers of C3b or C3b complexed with other complement proteins.

The surface assembly of terminal complement components was next examined. It has been postulated that for some gram-negative organisms, mechanisms contributing to serum resistance include inhibition of C5b-9 formation (25, 30, 31) and surface deposition of normal but nonlytic MAC (18–20, 35) or of altered, nonproductive complexes (14). C5b-9 was rapidly deposited on the surface of *B. pseudomallei*, with kinetics of deposition similar to that of C3. Also, as for C3, maximal deposition was observed only at the higher opsonizing serum concentrations. C5b-9 was detectable throughout the 60-min study period, suggesting that it was not shed from the bacterial surface as has been described for some *Salmonella* and *Aeromonas* species (19, 25).

Elution of C5b-9 in high-salt buffer has been used to examine the nature of the binding site for the MAC; resistance implies apparent attachment by strong hydrophobic interactions, whereas susceptibility to elution reportedly indicates that complexes are ionically bound to the bacterial surface (20). Our results demonstrating elution of C5b-9 suggest that a significant proportion of complexes are ionically bound to the bacterial surface, even in the presence of specific antibody. Although not all C5b-9 was eluted in 1 M NaCl, the proportion observed was similar to that reported for a serum-resistant, but not serum-sensitive, *Salmonella* strain (20). The relatively hydrophilic polysaccharide side chain has been identified as a potential site for the formation of a weak ionic binding site for C5b-9 (20). Interestingly, this would be consistent with the findings that the majority of C3 is ester bound indicating likely interaction with a bacterial polysaccharide moiety. Precise localization of surface-bound complement components by such methods as immunoelectron microscopy would assist in determining the mechanism of complement evasion should it occur in a distal nonmicrobicidal location. Furthermore, since bacteria may have more than one mechanism for evasion of complement-mediated lysis, other mechanisms such as abnormal C5b-9 assembly which have not been excluded in the present study may contribute to the serum resistance of *B. pseudomallei*.

Phagocytosis was significantly greater if bacteria were opsonized with PHS as compared with HHS, indicating that com-

plement is involved in cellular uptake. As the limitations of differential centrifugation may include failure to separate cell-associated noninternalized bacteria from ingested bacteria, internalization was examined by electron microscopy. It was highly suggestive of an intracellular location, as has been demonstrated by others (32, 34). Microbial deposition of C3b may result not only in C5 convertase assembly necessary for the formation of the MAC but, together with iC3b, serves as an opsonin for complement receptor-bearing phagocytes. Indeed, several intracellular pathogens have been shown to utilize complement receptor molecules to gain access to human phagocytic cells and establish infection (28, 36, 37). C3b-coated microorganisms will interact with the C3b receptor (CR1) (10), whereas iC3b-coated bacteria interact predominantly with complement receptor 3 (CR3) (11). The presence of covalently bound C3b and iC3b on the surface of *B. pseudomallei* indicates that interaction and/or subversion of CR1 and CR3 to gain entry to cells is possible. With complement receptor blockade, which has been used to demonstrate ligand-receptor uptake for other intracellular bacteria, namely, *Mycobacterium tuberculosis* (36), *Mycobacterium leprae* (37), *Legionella pneumophila* (28), and *Listeria monocytogenes* (9), we have demonstrated that both CR1 and CR3 are important for adherence to professional phagocytes (unpublished data).

A chemiluminescence response by PMNL, indicating that oxidative metabolites are produced, was observed in response to challenge with opsonized bacteria only. Chemiluminescence is not specific for ingestion since a positive response may also occur in response to cell surface-attached organisms, signaling via receptor sites. Nevertheless, we have demonstrated by a radiolabelled phagocytic assay together with examination by transmission electron microscopy that significant phagocytosis of opsonized bacteria by PMNL occurs. The kinetics of the oxidative response, which was not typical of the rapid respiratory burst observed during phagocytosis of other organisms such as *Staphylococcus aureus*, may be important for evasion of the oxidative microbicidal mechanisms of PMNL.

There exist conflicting reports as to the susceptibility of *B. pseudomallei* to the microbicidal activity of phagocytes. Razak and Ismail reported a 2-log reduction in bacterial viability following coincubation of *B. pseudomallei* with PMNL in the presence of 10% normal serum (34). In contrast, Pruksachartvuthi et al. found no significant killing of intracellular bacteria by human PMNL (32). Although some inhibition of growth of opsonized organisms was observed compared with that of unopsonized bacteria, our results demonstrating that *B. pseudomallei* was resistant to killing by PMNL concur with the latter findings. Further investigation of the mechanisms of this resistance is warranted as it may provide insights into the intracellular persistence of this intriguing, medically important organism.

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