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Intracellular Survival of *Burkholderia pseudomallei*

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***Burkholderia pseudomallei* is the causative agent of melioidosis, a disease being increasingly recognized as an important cause of morbidity and mortality in many regions of the world. Several features of melioidosis suggest that *B. pseudomallei* is a facultative intracellular pathogen. This study was designed to assess the ability of *B. pseudomallei* to invade and survive in eukaryotic cells. We have shown that *B. pseudomallei* has the capacity to invade cultured cell lines, including HeLa, CHO, A549, and Vero cells. We have demonstrated intracellular survival of *B. pseudomallei* in professional phagocytic cells, including rat alveolar macrophages. *B. pseudomallei* was localized inside vacuoles in human monocyte-like U937 cells, a histiocytic lymphoma cell line with phagocytic properties. Additionally, electron microscopic visualization of *B. pseudomallei*-infected HeLa cells and polymorphonuclear leukocytes confirmed the presence of intracellular bacteria within membrane-bound vacuoles. *B. pseudomallei* was found to be resistant to the cationic peptide protamine and to purified human defensin HNP-1.**

Burkholderia (*Pseudomonas*) *pseudomallei* is the causative agent of melioidosis (35). Melioidosis can be seen as an inapparent infection, asymptomatic pulmonary infiltration, acute localized suppurative infection, acute pulmonary infection (most common), acute septicemic infection, or chronic suppurative infection (7).

B. pseudomallei is a natural inhabitant of soil, stagnant water, and rice paddies in areas where it is endemic, which include Southeast Asia, particularly northeast Thailand and northern Australia (3, 32). Sporadic cases have also been reported in the Philippines, Mexico, Papua New Guinea, Africa, and South America. It is rare in the western hemisphere; however, a few cases have been reported in North America. These include laboratory-acquired infections and Vietnam veterans who developed the disease when they returned home (16, 25). This bacterium is a common cause of opportunistic infections in areas where it is endemic. Individuals particularly susceptible to infection include diabetics and those with renal disease (3). It has recently been shown that in some areas, this organism is a major cause of community-acquired sepsis, with a mortality which approaches 70% despite treatment (3).

B. pseudomallei produces several virulence factors, including endotoxin, exotoxin, and protease. *B. pseudomallei* also produces several other factors, such as lecithinase, lipase, hemolysin, and a water-soluble siderophore for iron acquisition from the host, which contribute to its survival and maintenance (1, 4, 36). Endotoxin has also been considered in the pathogenesis of the disease; however, the toxicity of *B. pseudomallei* endotoxin is reportedly low (4).

Several features of melioidosis suggest that *B. pseudomallei* is a facultative intracellular pathogen. The incubation period is not well defined and has ranged from 2 days to 26 years (26). This infection has the potential for a prolonged latency period, with recrudescence into an acute, fulminating, and fatal infection (23). The activation of the latent infection is often associated with concurrent disease or injury. Relapses of melioidosis after apparent clinical resolution of the primary infection have been assumed to be a recrudescence of the primary infection (22). Studies by Sexton et al. (32) and Desmarchelier et al. (9) have used ribotype analysis to demonstrate that, in many cases, relapse results from reactivation of a persistent endogenous source of infection. It has been postulated that after the initial phase of infection, *B. pseudomallei* can persist in a dormant stage in macrophages for months or years (27). *B. pseudomallei* has been found to survive and multiply in human phagocytes in vitro (27). The mechanism by which this organism survives within human phagocytes is not known.

Relapse after apparent cure, frequent recurrences of infection when the course of treatment is not long enough, and difficulty in treating melioidosis despite the fact that antimicrobial agents are effective against the organism in vitro also suggest that intracellular survival of *B. pseudomallei* may be important in the pathogenesis of infection (19, 27).

The present studies were designed to examine the ability of *B. pseudomallei* to invade nonprofessional phagocytic cells, to assess intracellular survival and replication in professional phagocytes, and to investigate possible mechanisms by which *B. pseudomallei* may survive intracellularly.

MATERIALS AND METHODS

Bacterial strains. *B. pseudomallei* 316c was generously donated by D. A. B. Dance, Oxford Tropical Medicine Research Program, Wellcome-Mahidol University, Bangkok, Thailand. *Salmonella typhimurium* 14028s was provided by K. Sanderson, Salmonella Genetic Stock Center, University of Calgary, Calgary, Alberta, Canada. *Escherichia coli* HB101 was used in the cell invasion assays, and *E. coli* ATCC 25922 was used in the protamine sensitivity assays. Bacterial strains were grown on Luria broth agar or tryptic soy agar (TSA), and broth cultures were grown in Luria broth (LB) or tryptic soy broth (TSB) overnight at 37°C with shaking at 200 rpm. Overnight cultures of bacterial strains were subcultured 1:20 to 1:30 in TSB and grown for an additional 5 h.

Culture of cell lines. Cell lines were obtained from the American Type Culture Collection (Rockville, Md.). All cell lines were maintained in cell culture medium containing a standard antibiotic mixture (100 U of penicillin, 0.1 mg of streptomycin, and 0.25 μg of amphotericin B per ml [Sigma Chemical Co., St. Louis, Mo.]) plus 10% fetal bovine serum (FBS; Gibco Canada Inc., Mississauga, Ontario, Canada) at 37°C in 5% CO_2. The culture medium for cell lines HeLa, A549, and Vero was Dulbecco's modified Eagle's medium (DMEM). CHO cells were maintained in DMEM containing nutrient mixture F12 (DMEM/F-12), and

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TABLE 1. Internalization of *B. pseudomallei*, *S. typhimurium*, and *E. coli* into cultured epithelial and fibroblast cell lines, as measured by the kanamycin protection assay[a]

Cells	*B. pseudomallei* 316c		*S. typhimurium* 14028s		*E. coli* HB101	
	Inoculum (no. of cells)	Mean no. of intracellular organisms	Inoculum (no. of cells)	Mean no. of intracellular organisms	Inoculum (no. of cells)	Mean no. of intracellular organisms
HeLa	4.2×10^6	$4.8 \times 10^4 \pm 2.0 \times 10^4$[b]	2.0×10^7	$1.3 \times 10^6 \pm 6.4 \times 10^5$	8.8×10^6	33 ± 57
Vero	9.8×10^6	$4.0 \times 10^4 \pm 1.1 \times 10^4$[b]	2.0×10^7	$5.7 \times 10^5 \pm 1.6 \times 10^5$	3.4×10^6	66 ± 115
A549	1.0×10^7	$1.1 \times 10^4 \pm 3.0 \times 10^3$[b]	1.8×10^7	$7.2 \times 10^5 \pm 3.6 \times 10^5$	4.0×10^6	0.0 ± 0.0
CHO	9.8×10^6	$5.3 \times 10^5 \pm 1.5 \times 10^5$[b]	2.2×10^7	$4.4 \times 10^6 \pm 8.2 \times 10^5$	4.7×10^6	$1.2 \times 10^3 \pm 2.5 \times 10^2$

[a] 5×10^5 tissue culture cells were incubated with 25 μl of bacteria diluted to give an MOI of approximately 10 bacteria per eukaryotic cell. Bacteria were incubated with the cultured cell lines for 2 h at 37°C in 5% CO_2 to allow bacterial entry, followed by an additional 2-h incubation in medium containing kanamycin (100 μg/ml) to kill extracellular bacteria. Eukaryotic cells were then lysed with 0.1% Triton X-100, and intracellular bacteria were quantitated by plating serial dilutions of the lysate. Data are expressed as the mean ± standard error of the mean (SEM) for three wells.

[b] $P < 0.05$ compared with noninvasive *E. coli* HB101.

U937 cells were maintained in RPMI 1640. All cell culture media were obtained from Gibco Canada Inc.

Cell invasion assay. The ability of *B. pseudomallei* 316c, *S. typhimurium* 14028s, and *E. coli* HB101 to invade various epithelial and fibroblast cell lines was examined. A549, CHO, Vero, and HeLa cells were seeded in 24-well plates at 5×10^5 cells per well in the appropriate medium plus 10% FBS and grown overnight. Invasion assays were performed by the methods of Elsinghorst (11). Briefly, monolayers were washed twice in phosphate-buffered saline (PBS), and 1 ml of fresh medium was added to the monolayers. Washed bacteria were diluted to the appropriate concentration in tissue culture medium, and 25 μl of bacteria was added to the monolayers. After 2 h at 37°C, the monolayers were washed with PBS, and 1.5 ml of culture medium containing kanamycin (100 μg/ml) was added to the monolayers for an additional 2 h to kill extracellular organisms. The monolayers were washed with PBS and lysed with 0.1% Triton X-100 (BDH Chemicals, Toronto, Ontario, Canada). Intracellular bacteria were quantitated by plating serial dilutions of the lysate on TSA plates. All invasion assays were performed in triplicate.

B. pseudomallei-infected HeLa cells and uninfected cells were prepared for electron microscopy by the methods of Beveridge et al. (2). Cells were washed three times with 0.1 M phosphate (pH 7.2), fixed for 1 h in 2.5% phosphate-buffered glutaraldehyde (EM Sciences, Fort Washington, Pa.), and postfixed in 1% phosphate-buffered osmium tetroxide (EM Sciences). After washing, the cells were stained in 2% uranyl acetate (Fisher Scientific), washed again, and dehydrated through an ethanol-propylene oxide series to Epon 812 (Can EM, Guelph, Canada). The polymerized blocks were thin sectioned, and the sections were mounted on Formvar carbon-coated 200-mesh copper grids and stained with uranyl acetate and lead citrate. The sections were viewed in a Philips EM 300 operating at 60 kV under standard conditions with the cold finger in place. Electron microscopy was performed at the scanning and transmission electron microscope (STEM) Facility at the University of Guelph, Guelph, Ontario, Canada.

To determine if uptake of *B. pseudomallei* into cultured epithelial cells was inhibitable by cytochalasin D, A549 cells were preincubated with 1 μg of cytochalasin D (Sigma Chemical Co.) per ml for 30 min prior to the addition of bacteria by the method of Rosenshine et al. (30). The cell invasion assay was performed as previously described except that cytochalasin D was kept in the medium throughout the invasion assay. *S. typhimurium* was used as a control, as uptake of this organism into cultured epithelial cells has been shown previously to be inhibitable by cytochalasin D (30).

U937 cell uptake of *B. pseudomallei*. The ability of human monocyte-like U937 cells to ingest *B. pseudomallei* 316c was assessed by a modification of the methods of Elsinghorst (11). Briefly, U937 cells were diluted to 2×10^5 cells per ml in RPMI 1640 and incubated with 100 μl of bacteria which had been diluted to 10^8 CFU/ml for 2 h at 37°C in 5% CO_2. U937 cells were washed with PBS and incubated for an additional 2 h with 1 ml of RPMI 1640 containing kanamycin (100 μg/ml). *B. pseudomallei*-infected U937 cells and uninfected cells were prepared for electron microscopy as previously described.

Intracellular survival and replication of *B. pseudomallei* in rat alveolar macrophages. The ability of *B. pseudomallei* 316c to survive and replicate in rat alveolar macrophages over a 20-h period was assessed. Six 350-g male Sprague-Dawley rats (Charles River) were killed; the trachea was exposed, and a blunt 18-gauge needle was inserted and secured with surgical silk. The lungs were lavaged once with 5 ml of Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+}, which was then discarded. The lungs were then lavaged five times with 7 ml of PBS. Alveolar macrophages from the pooled lavage fluid were centrifuged ($800 \times g$), resuspended in RPMI 1640, and counted with a hematocytometer. Alveolar macrophages were adjusted to 2×10^5 cells per ml. Bacteria were washed and adjusted to 10^7 CFU/ml. Entry of *B. pseudomallei* into alveolar macrophages after 4 h of incubation was determined as described above for U937 cells. The survival of *B. pseudomallei* in alveolar macrophages over a 20-h incubation was determined in duplicate cultures. After killing of extracellular bacteria with kanamycin, the cells were washed with RPMI 1640 and incubated for an additional 16 h in RPMI 1640 containing kanamycin (20 μg/ml) to inhibit the growth of any remaining extracellular organisms. After a total incubation of 20 h, the macrophages were lysed, and intracellular organisms were quantitated as described above. The viability of the macrophages was determined at 4 and 20 h by trypan blue dye exclusion.

Intracellular survival and replication of *B. pseudomallei* in human PMN. By the methods of Rest and Speert (28), polymorphonuclear leukocytes (PMN) were isolated from heparinized whole blood from healthy donors by Ficoll-Hypaque (Lymphoprep; C-6 Diagnostics, Mequon, Wis.) density gradient centrifugation. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophil viability was determined by trypan blue exclusion. Neutrophils were diluted to 10^7 cells per ml in RPMI 1640; 500 μl of neutrophils was incubated with 100 μl of bacteria diluted to 5×10^9 cells per ml and 100 μl of autologous normal human serum. Entry of *B. pseudomallei* into PMN and intracellular survival over 20 h were determined as described above for rat alveolar macrophages.

B. pseudomallei-infected and uninfected PMN were prepared for electron microscopy as described above.

Effects of incubation in normal human serum on *B. pseudomallei* survival in PMN. By the methods of Kalmar (20), *B. pseudomallei* 316c was incubated in 100 μl of autologous nonimmune serum diluted 1:2 in RPMI 1640 for 15 min at 37°C with constant agitation. Control organisms were incubated with 100 μl of RPMI 1640 under identical conditions. Organisms incubated with normal human serum and control organisms were added to PMN, and uptake and intracellular survival of *B. pseudomallei* were determined as described above.

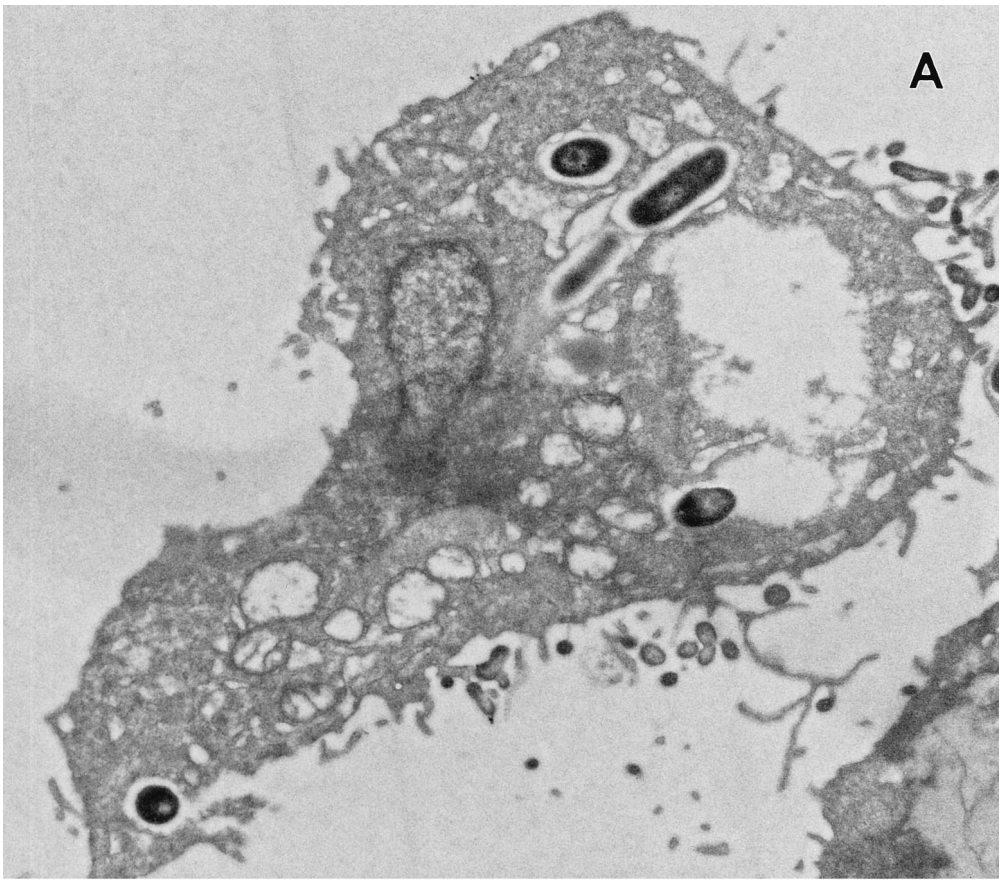
Growth assays with protamine sulfate. The effects of protamine on bacterial growth were examined. *B. pseudomallei* 316c, *S. typhimurium* 14028s, and *E. coli* ATCC 25922 were grown in M9 minimal medium (29) plus 0.5% glucose and various concentrations of protamine sulfate (Sigma Chemical Co.). Strains were cultured from frozen stock onto M9 agar (29) containing 0.5% glucose. A stock solution of 10-mg/ml protamine sulfate was made in water and filter sterilized with a 0.22-μm filter. Flasks containing M9 broth plus various concentrations of protamine sulfate were inoculated with 100 μl of an overnight starter culture. In control cultures, bovine serum albumin was added to a final protein concentration of 1 mg/ml. These growth experiments were performed in triplicate, and the growth rate was determined by measuring the A_{595} of the cultures at 24 h following inoculation. All cultures were incubated at 37°C in a shaking (200 rpm) incubator.

Antimicrobial activity of HNP-1 against *B. pseudomallei*. The bactericidal activity of human neutrophil peptide 1 (HNP-1) was tested against *B. pseudomallei* 316c, *S. typhimurium* 14028s, and *E. coli* HB101 under standard assay conditions by the methods of Harwig et al. (18). HNP-1 was generously provided by M. Selsted, University of California, Irvine. HNP-1 was stored as a 1-mg/ml stock solution in 0.01% acetic acid at −20°C. Cells were washed in 10 mM phosphate and adjusted to 5×10^6 CFU/ml. Bacteria were incubated in 10 mM phosphate buffer containing HNP-1 (50 μg/ml) or an equivalent volume of 0.01% acetic acid as a control. After 2 h at 37°C, the reactions were stopped by the addition of 0.15 M NaCl. The number of CFU was determined by plating serial dilutions on TSA. Each incubation was performed in triplicate.

Statistical analysis. Data were analyzed for statistical significance by the paired *t* test.

RESULTS

Invasion of cultured cell lines. *B. pseudomallei* 316c is a blood culture isolate from a septicemic melioidosis patient. *S. typhimurium* 14028s was used as a virulent, invasive control, and *E. coli* HB101 was used as a noninvasive control. *B.*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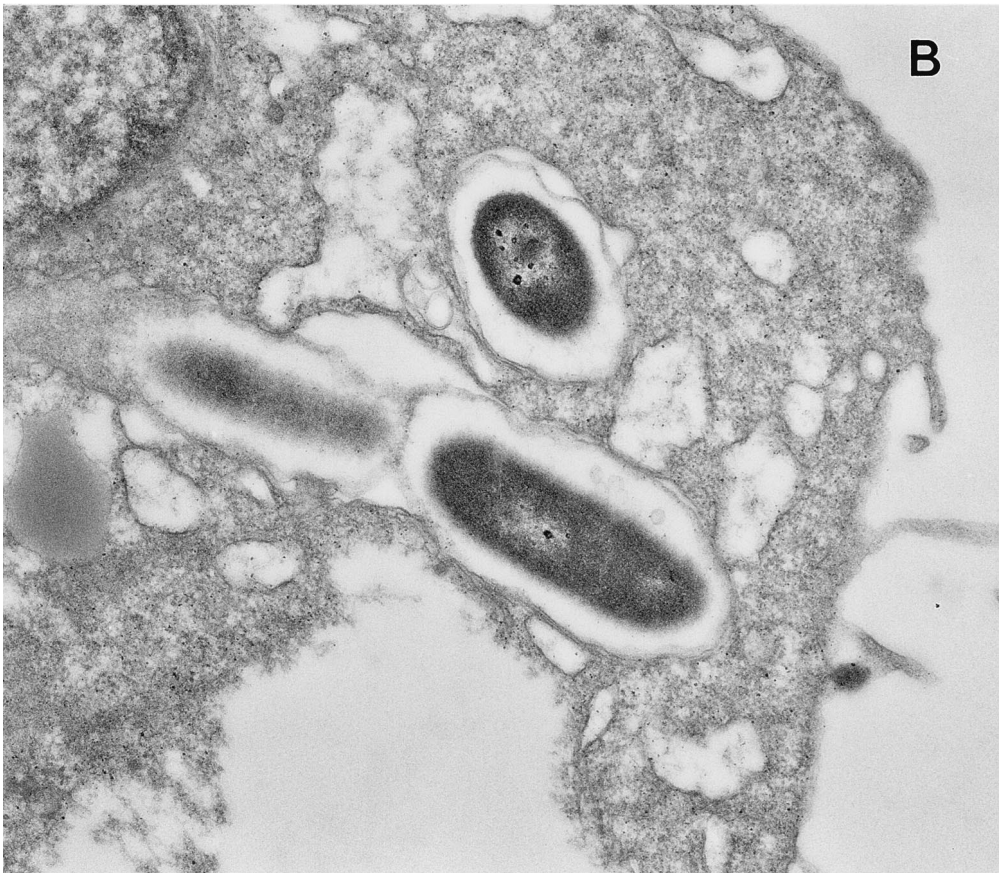
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FIG. 1. Transmission electron micrographs of *B. pseudomallei* 316c-infected HeLa cells at 4 h postinfection. HeLa cells were incubated with *B. pseudomallei* 316c at an MOI of 10 bacteria per HeLa cell for 2 h at 37°C. Extracellular bacteria were killed by incubating the infected HeLa cell monolayers with kanamycin (100 μg/ml) for an additional 2 h at 37°C. Infected monolayers were then prepared for electron microscopy. (A and B) *B. pseudomallei*-infected HeLa cell containing intracellular organisms at (A) magnification ×7,290 and (B) magnification ×47,448.

pseudomallei was able to enter three epithelial cell lines and one fibroblast cell line (Table 1). In all cases, *B. pseudomallei* was less invasive than the virulent *S. typhimurium* 14028s. At a multiplicity of infection (MOI) of 10 bacteria per eukaryotic cell, the number of *B. pseudomallei* found in HeLa cells was significantly different from that of the noninvasive control, *E. coli* HB101. For *B. pseudomallei*, 1.1% of the inoculum entered the cells, compared with 6.5% of the *S. typhimurium* inoculum and 0% of the *E. coli* inoculum. The ability of *B. pseudomallei* to invade HeLa cells was also examined at MOIs of 1 and 100. In each case, *B. pseudomallei* entered HeLa cells in numbers significantly different from those of *E. coli* HB101 (data not shown).

These data, suggesting the intracellular presence of viable *B. pseudomallei*, were confirmed by electron microscopic examination of infected HeLa cells (Fig. 1). *B. pseudomallei* was present inside HeLa cells in membrane-bound vacuoles.

At an MOI of approximately 10, the number of *B. pseudomallei* found intracellularly in A549 cells was significantly different from that in the *E. coli* control. For *B. pseudomallei*, 0.11% of the inoculum entered the cells, compared with 4% of the *S. typhimurium* inoculum and 0% of the *E. coli* inoculum. At an MOI of 10, the number of *B. pseudomallei* found in CHO cells was significantly different from that in the *E. coli* control. For *B. pseudomallei*, 5.4% of the inoculum entered the cells, compared with 20% of the *S. typhimurium* inoculum and 0.03% of the *E. coli* inoculum. In Vero cells, a fibroblast cell line, at an MOI of 10, the number of *B. pseudomallei* found intracellularly was also significantly different from that for *E. coli*. For *B. pseudomallei*, 0.4% of the inoculum entered the cells, compared with 2.9% of the *S. typhimurium* inoculum and 0% of the *E. coli* inoculum.

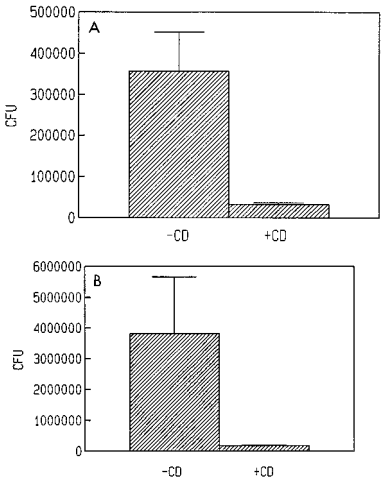


FIG. 2. Effect of cytochalasin D (CD) on internalization of *B. pseudomallei* 316c. A549 cell monolayers were treated with cytochalasin D (1 μg/ml) for 30 min. *S. typhimurium* 14028s or *B. pseudomallei* 316c was then added to the monolayers at an MOI of 10 and incubated for 2 h at 37°C, and extracellular organisms were killed by an additional 2-h incubation with kanamycin (100 μg/ml). Cell monolayers were then lysed, and viable intracellular bacteria were quantitated. Cytochalasin D was kept in the incubation medium throughout the experiment. (A) *B. pseudomallei* 316c. (B) *S. typhimurium* 14028s. Note the 10-fold difference in the scales representing bacterial CFU in panels A and B. Data are expressed as mean number of CFU ± standard error of the mean.

The invasion of A549 cells by *B. pseudomallei* 316c was found to be inhibitable by cytochalasin D (Fig. 2A). Incubating A549 cells with cytochalasin D for 30 min prior to the addition of bacteria reduced the invasion of *B. pseudomallei* to 9% of that seen in A549 cells incubated without cytochalasin D. Similarly, the invasion of cytochalasin D-treated A549 cells by *S. typhimurium* 14028s was reduced to 4% of the control level (Fig. 2B).

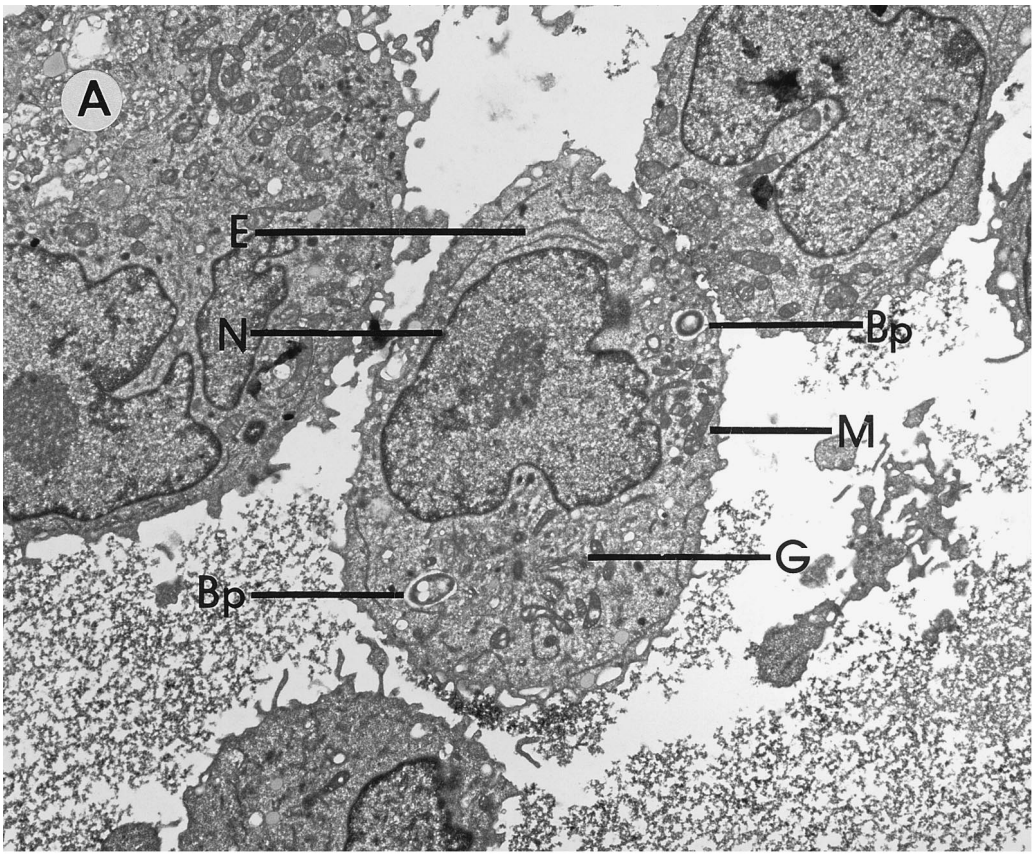
U937 cell uptake of *B. pseudomallei*. Figure 3 shows transmission electron micrographs of *B. pseudomallei* 316c-infected U937 cells. Numerous intracellular bacteria were seen in *B. pseudomallei*-infected U937 cells. Individual *B. pseudomallei* organisms were seen in intact membrane-bound vacuoles within the cytoplasm of infected U937 cells.

Intracellular survival and replication of *B. pseudomallei* in rat alveolar macrophages. *B. pseudomallei* was shown to survive and replicate inside rat alveolar macrophages. A total of $1.3 \times 10^4 \pm 6.2 \times 10^3$ organisms, or 1.3% of the inoculum, were located intracellularly in rat alveolar macrophages after 4 h. After an additional 16 h of incubation, the number of organisms located intracellularly increased to $3.4 \times 10^6 \pm 2.1 \times 10^6$, which represented a statistically significant 2-log-unit increase ($P < 0.05$). In all cases, macrophage viability at both time points exceeded 95%, as determined by trypan blue exclusion.

Intracellular survival and replication of *B. pseudomallei* in PMN. After the initial 4-h incubation period, $8.6 \times 10^5 \pm 3.0 \times 10^5$ organisms were located intracellularly in PMN, which represents 1.4% of the inoculum. After an additional 16 h of incubation, the number of intracellular organisms increased to $7.0 \times 10^6 \pm 1.7 \times 10^6$, which represented a statistically significant increase of approximately ninefold ($P < 0.05$). The viability of the PMN at both 4 and 20 h exceeded 95%, as determined by trypan blue exclusion. Figure 4 shows transmission electron micrographs of *B. pseudomallei*-infected and control PMN at 4 h postinfection. Numerous intracellular bacteria were visible in many of the infected PMN. *B. pseudomallei*-infected PMN were seen to have membrane-bound vacuoles containing both individual organisms and groups of organisms. In some cases, the membrane of the *B. pseudomallei*-containing vacuole appeared to be in the process of degenerating (Fig. 4). For most bacteria killed by PMN, 99.9% of the killing occurs within the first hour of culture, and a 20-h incubation with PMN results in data of questionable significance. It is likely that this is the case with these studies on *B. pseudomallei*, and the limitations of the 20-h incubation should be recognized.

The effects of incubation in normal human serum on the entry and intracellular survival of *B. pseudomallei* were determined. Incubation of bacteria with normal human serum prior to exposure to the PMN did not significantly affect uptake or intracellular survival (data not shown).

Effects of protamine on bacterial growth. The effects of protamine sulfate on bacterial growth are shown in Fig. 5. Protamine is a 32-amino-acid cationic peptide from salmon sperm that has been shown to preferentially kill peptide-sensitive salmonellas (17). Protamine is often used as an indicator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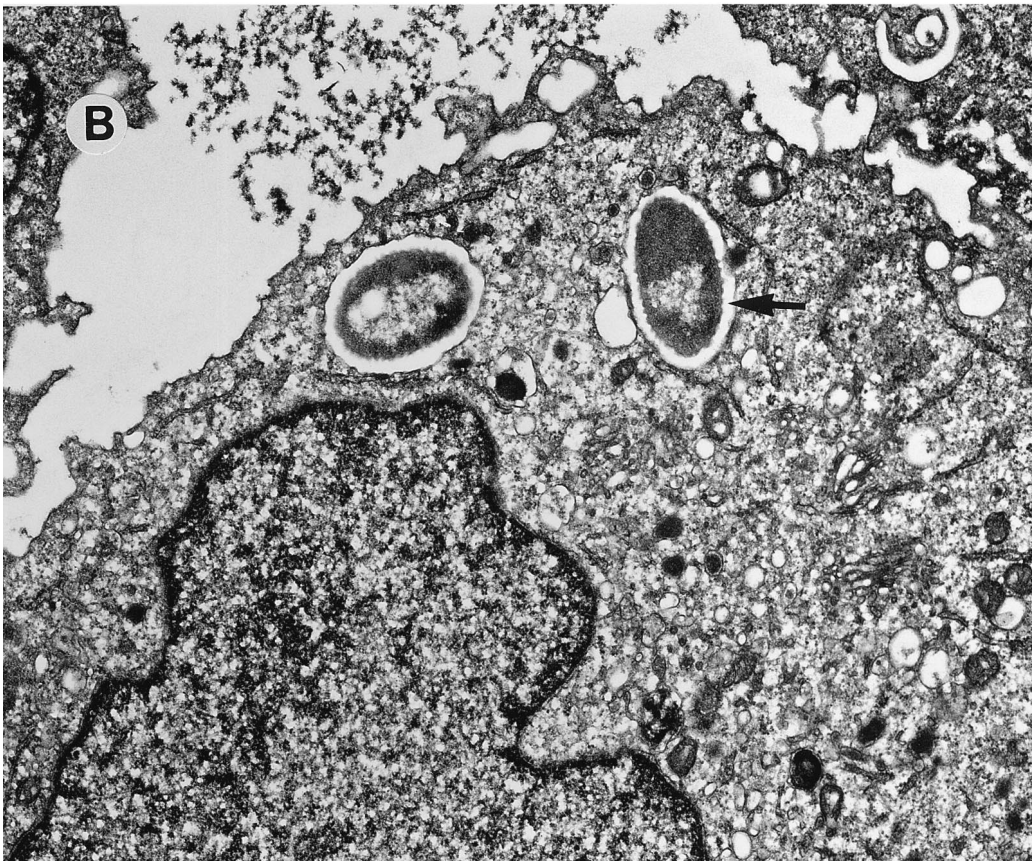
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TABLE 2. Antimicrobial activity of human defensin HNP-1[a]

Strain	Mean $\log_{10}$ CFU			Log_{10} decrease in CFU/ml
	Inoculum	Control (0.01% HoAc)	+ HNP-1 (50 μg/ml)	
S. typhimurium 14028s	6.85	7.22	6.98	0.24
B. pseudomallei 316c	6.17	6.49	6.91	−0.42*
E. coli HB101	6.35	6.34	5.51	0.83*

[a] Incubations were conducted with overnight cultures, subcultured 1:30 and grown for an additional 5 h in TSB. Bacteria were incubated with HNP-1 or an equivalent volume of 0.01% acetic acid (HoAc) in 10 mM phosphate buffer (pH 7.4) containing 1% TSB for 2 h at 37°C. Values represent $\log_{10}$ mean CFU of triplicate assays and $\log_{10}$ decrease in CFU relative to the control incubated with 0.01% acetic acid for 2 h. *, $P < 0.05$ compared with bacteria incubated with 0.01% acetic acid.

of susceptibility to physiologically relevant compounds such as defensins, magainins, and cecropins (17). *E. coli* was unable to grow at any concentration of protamine tested. *S. typhimurium* showed moderate growth at 100 μg/ml and minimal growth at higher concentrations of protamine. The growth of *B. pseudomallei* 316c was unaffected by any concentration of protamine.

Antimicrobial effects of HNP-1. The effects of the human defensin HNP-1 on *B. pseudomallei* 316c and the control strains are shown in Table 2. Individual purified defensins HNP-1 and HNP-3 have been shown to be as active as a mixture of HNP-1, HNP-2, and HNP-3 (13). *S. typhimurium* 14028s was not significantly affected by exposure to HNP-1, an observation consistent with previous studies in which this strain was shown to be peptide resistant (17). HNP-1 was significantly bactericidal for *E. coli* HB101, producing a 0.83 $\log_{10}$ decrease in viable CFU ($P < 0.05$). Under the conditions used in our assays, *B. pseudomallei* showed a statistically significant increase in CFU after incubation with HNP-1 ($P < 0.05$). The viable CFU of *B. pseudomallei* increased 0.42 $\log_{10}$ unit during exposure to HNP-1.

DISCUSSION

The latent form of melioidosis is of considerable diagnostic and epidemiological importance. Activation of a latent infection often results in an acute, fulminating, and fatal infection and is frequently associated with concurrent disease or injury (3, 23). This often leads to difficulty in prompt diagnosis, and in conjunction with the resistance to antimicrobial therapy encountered in chronic forms of melioidosis, this infection often results in significant morbidity and mortality (7, 33). The ability of *B. pseudomallei* to survive and multiply in both professional and nonprofessional phagocytes may provide an explanation for both the occurrence of latent infections and the relapses of infection that result from reactivation of a persistent endogenous source of infection.

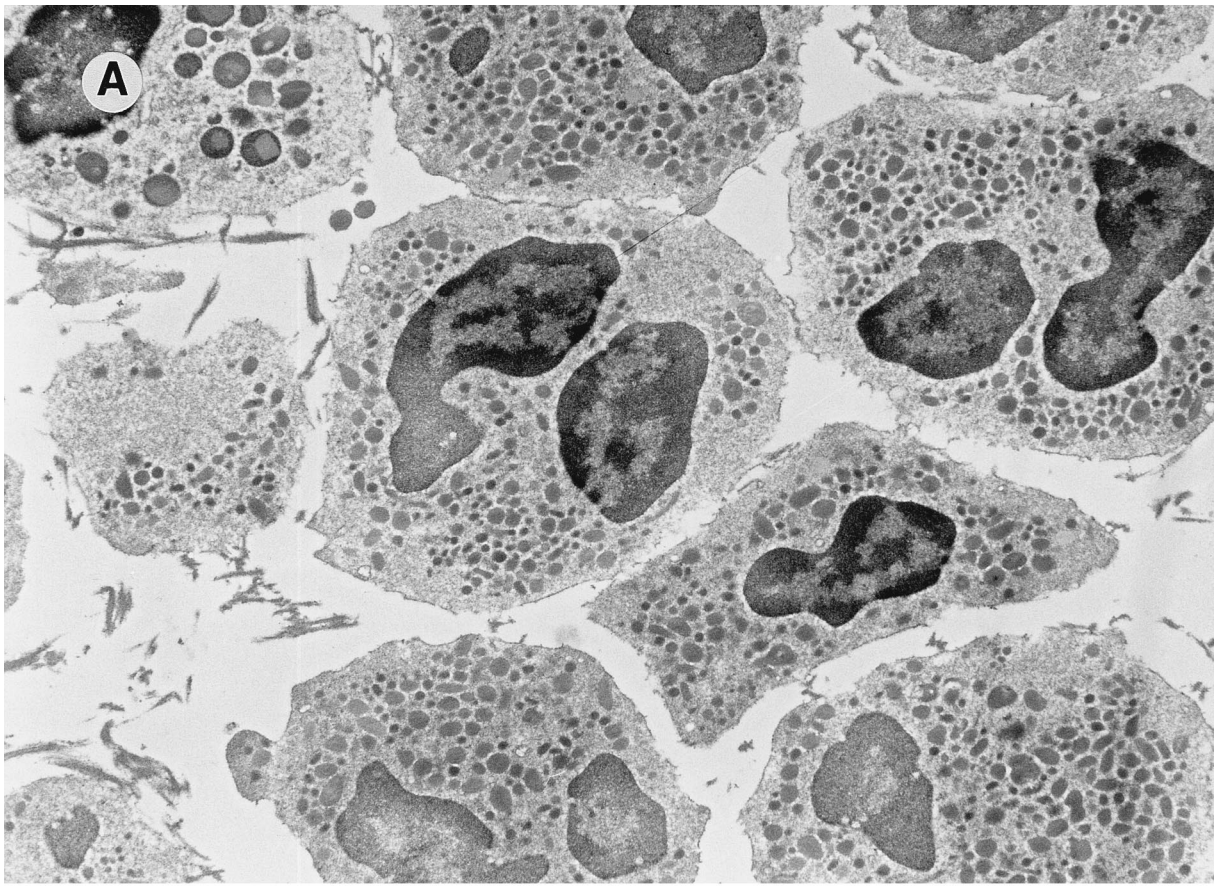
In the current study, we have demonstrated the ability of *B. pseudomallei* to invade several cultured cell lines that are normally nonphagocytic, including both epithelial cells and fibroblasts. The human pulmonary carcinoma cell line A549 possesses the morphological and biochemical characteristics of type II pneumocytes of the intact lung (24). The interaction of *B. pseudomallei* with A549 cells, an established pulmonary epithelial cell line, provides a means to study the early phase of human respiratory tract infection by this organism. Entry of *B. pseudomallei* into these cultured cell lines occurs at a slightly lower level than that seen for virulent, invasive *S. typhimurium* 14028s. This is the first report describing the ability of *B. pseudomallei* to enter nonprofessional phagocytic cells.

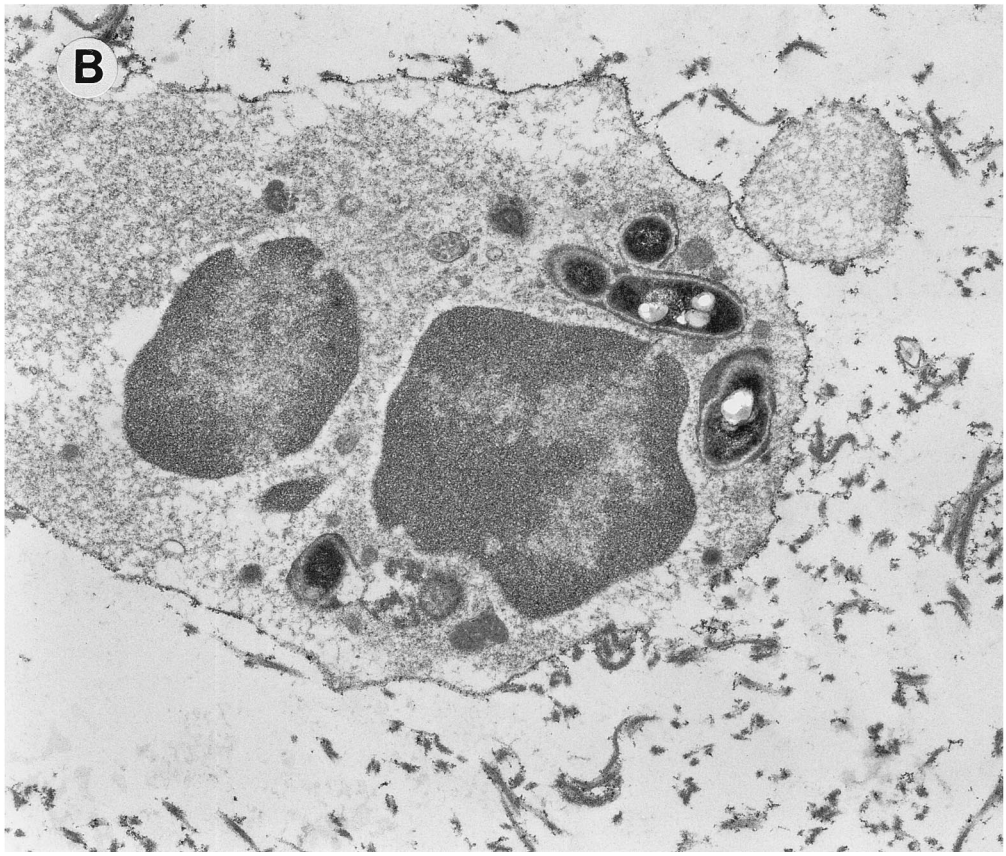
B. pseudomallei entry into A549 cells was inhibitable with cytochalasin D. Cytochalasins inhibit actin polymerization, thereby blocking microfilament function. Cytochalasin D is considered the most specific of the cytochalasins (5, 10). Bacterial invasion usually involves exploitation of host cell functions to facilitate invasion; thus, the uptake of many invasive organisms, including *E. coli* and *Salmonella*, *Shigella*, and *Yersinia* species can be blocked by cytochalasins (30). The inhibition of invasion of *B. pseudomallei* into cultured epithelial cells by cytochalasin D suggests that, like other invasive organisms, *B. pseudomallei* makes use of host cell components, specifically microfilaments, to facilitate invasion into eukaryotic cells.

Intracellular survival and replication of *B. pseudomallei* in professional phagocytic cells, including alveolar macrophages, were demonstrated. The ability of microorganisms to survive within phagocytic cells is a potent method of preventing clearance from the host (34). Intracellular pathogens have adopted various strategies to evade host defense mechanisms, including invasion of nonprofessional phagocytes, escape into the cytoplasmic compartment, interference with reactive oxygen intermediates, inhibition of phagosome-lysosome fusion and phagosomal acidification, and resistance to lysosomal contents and cationic peptides such as defensins (21).

Defensins are a family of cationic peptides, 3 to 4 kDa (29 to 34 amino acids), found in human, rabbit, rat, and guinea pig neutrophils, rabbit alveolar macrophages, and mouse small intestinal (Paneth) cells (13, 14). No defensin peptides in human macrophages have been reported (6). Under the conditions used in our assays, *B. pseudomallei* 316c showed a statistically significant increase in viable CFU during exposure to the purified human defensin HNP-1. To our knowledge, this is the first report of an organism showing significant growth in the presence of defensins. *S. typhimurium* 14028s was not significantly affected by exposure to HNP-1, an observation consistent with previous studies showing that this strain is considered peptide resistant (17). As previously reported by Ganz et al. (14), *E. coli* was effectively killed by HNP-1. The resistance of bacteria to intracellular killing by neutrophil defensins may facilitate their eventual escape from the short-lived neutrophil, allowing their subsequent encounter with monocytes that arrive later at the site of microbial invasion (8). In this manner,

FIG. 3. Transmission electron micrographs of intracellular *B. pseudomallei* 316c within U937 cells. U937 cells were incubated with *B. pseudomallei* 316c at an MOI of 100 bacteria per U937 cell for 2 h at 37°C. Extracellular bacteria were killed by incubating the infected U937 cells with kanamycin (100 μg/ml) for an additional 2 h at 37°C. Infected U937 cells were then prepared for electron microscopy. (A) *B. pseudomallei*-infected U937 cell at 4 h postinfection, showing major cellular structures and intracellular bacteria within membrane-bound vacuoles. Bp, *B. pseudomallei*; N, nucleus; E, endoplasmic reticulum; G, Golgi apparatus; M, mitochondria. Magnification, ×11,540. (B) *B. pseudomallei*-infected U937 cell at 4 h postinfection. The arrow indicates *B. pseudomallei* in a membrane-bound vacuole. Magnification, ×33,450.


A


B

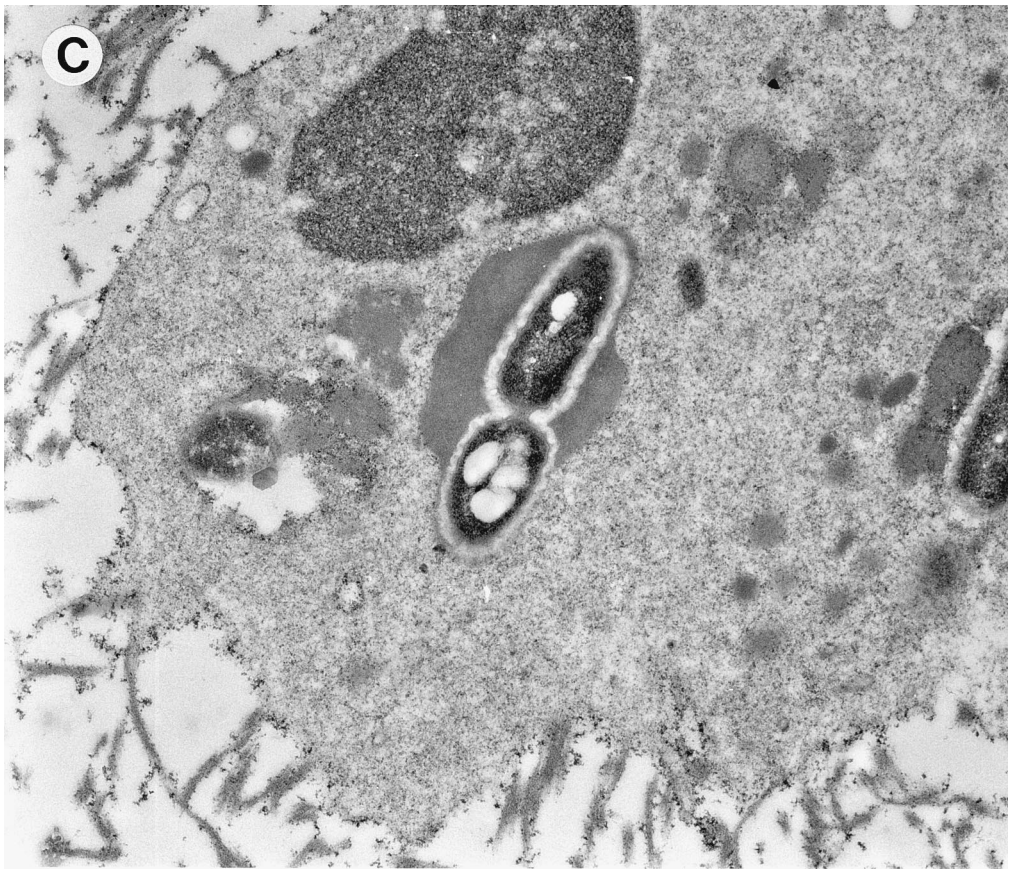


FIG. 4. Transmission electron micrographs of *B. pseudomallei* 316c within human PMN. Normal human PMN were isolated from whole blood and incubated with *B. pseudomallei* 316c at an MOI of 100 bacteria per PMN for 2 h at 37°C. Extracellular bacteria were killed by incubating the infected PMN with kanamycin (100 μg/ml) for an additional 2 h at 37°C. Control and infected PMN were then prepared for electron microscopy. (A) Noninfected PMN. Magnification, ×10,386. (B) Infected PMN at 4 h postinfection, containing numerous intracellular bacteria. Magnification, ×23,553. (C) Infected PMN at 4 h postinfection. Magnification, ×37,775.

the resistance of *B. pseudomallei* to the antimicrobial activity of defensins may facilitate intracellular survival.

Resistance to antimicrobial peptides was first correlated with virulence in *S. typhimurium* (12). In *S. typhimurium*, the integrity of the *phoPQ* operon is required for resistance to rabbit defensin NP-1, virulence in mice, survival within macrophages, and resistance to low pH (17). PhoP and PhoQ are highly similar in sequence to the family of two-component regulatory systems. PhoP has significant sequence homology to response regulators, and PhoQ has significant sequence homology to sensor kinases (15). None of the known PhoP-regulated genes have been shown to play a direct role in defensin resistance (17).

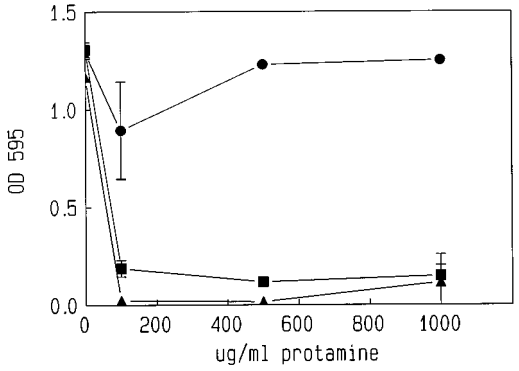


FIG. 5. Effects of protamine on the growth of *B. pseudomallei* 316c, *S. typhimurium* 14028s, and *E. coli* ATCC 25922. Strains were grown in M9 minimal salts medium with glucose (0.5%) as a carbon source and various concentrations of protamine. After 24 h of growth at 37°C in a shaking incubator, bacterial growth was examined by measuring the absorbance of the cultures at 595 nm. ●, *B. pseudomallei* 316c; ■, *S. typhimurium* 14028s; ▲, *E. coli* ATCC 25922.

The clinical manifestations of melioidosis and its protracted course have led researchers to suspect that *B. pseudomallei* is a facultative intracellular pathogen capable of growth within eukaryotic cells. We have clearly demonstrated that *B. pseudomallei* has the ability to both invade nonprofessional phagocytes and survive intracellularly in professional phagocytic cells. We have begun to investigate the possible mechanism of intracellular survival of *B. pseudomallei*. Resistance to defensins may contribute to intracellular survival of *B. pseudomallei* by initially allowing the organisms to escape killing by neutrophils. *B. pseudomallei* would then be able to interact with less bactericidal mononuclear phagocytes. *B. pseudomallei* was seen in membrane-bound vacuoles within all cell types examined by electron microscopy. In the current study, we were unable to determine if phagosome-lysosome fusion was occurring in the eukaryotic cells, and studies are under way to assess phagosome-lysosome fusion in *B. pseudomallei*-infected cells. Several of the membranes of vacuoles containing *B. pseudomallei* in infected cells appeared to be degenerating. This may

represent escape of the organisms from the phagosome. Additional studies to confirm this observation are under way.

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