

An electronmicroscopic study of the interaction of *Burkholderia pseudomallei* and human macrophages

Sakthi A NATHAN *BSc, MMed Sc*, and SD PUTHUCHEARY *MBBS, FRCPath*

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia

Abstract

B. pseudomallei has been shown to persist intracellularly in melioidosis patients until reactivated by decreasing immunocompetence. We have shown by transmission electron microscopy the internalization of *B. pseudomallei* by human macrophages via conventional phagocytosis enclosed within membrane-bound vacuoles or phagosomes. Ferritin labeled lysosomes provided evidence of phagosome-lysosome fusion. Ingested bacilli were designated as “intact” or “damaged” on the basis of their ultrastructural features. An intact bacterium was seen with low electron opaque central nuclear region surrounded by dense bacterial cytoplasm, bounded externally by bacterial plasma membrane and cell wall. In contrast, *B. pseudomallei* were considered damaged when seen with cavitation within the central nuclear region, separation of bacterial cytoplasm from the cell wall, herniation of cytoplasmic contents and lamination of bacterial cell wall and its surrounding electron transparent zone. Our observations indicate that the microbicidal mechanism(s) in *B. pseudomallei*-infected macrophages failed to ensure complete clearance of the organism and this failure probably facilitates intracellular persistence and proliferation, and this may be one of the survival strategies adopted by this organism.

Key words: *B.pseudomallei*, human macrophages, intracellular survival

INTRODUCTION

Melioidosis is an infectious disease of humans and animals caused by *Burkholderia pseudomallei*, a gram-negative saprophyte found in soil and surface water of endemic areas. Infection is acquired by contact with contaminated soil and water and by inhalation of dust particles. Clinical manifestations of human melioidosis are extremely variable and range from subclinical infections to fulminant septicaemia with a high mortality rate.¹ Relapse and recurrence in melioidosis is often associated with concurrent disease or injury despite prolonged and appropriate antimicrobial therapy. This is usually the result of reactivation of a persistent endogenous source.² Intracellular replication and localization of the bacterium has been documented in professional phagocytic³ and non-phagocytic cell lines.⁴ The interaction of host cells with *B. pseudomallei* has been extensively studied using cell lines such as mouse macrophage cell line RAW 264.7, Hela cells and human macrophage-like cell line U937.⁴ In this study, using transmission electron microscopy,

we have attempted to show the sequence of events that take place intracellularly when opsonised *B. pseudomallei* are ingested by human macrophages and have demonstrated intact and probably viable organisms remaining inside these cells.

MATERIALS AND METHODS

Bacterial isolate. *B. pseudomallei* was isolated from a septicaemic patient on antimicrobial therapy, at the University Malaya Medical Centre, Kuala Lumpur and identified using routine laboratory methods and confirmed by the API 20 NE system (bioMeriux, France). The culture was stored in brain heart infusion broth at -70°C and subcultured via blood agar to nutrient broth (Becton Dickinson, USA) and incubated at 37°C for 16 hours.

Isolation of human mononuclear cells. Heparinized blood sample was obtained from a healthy donor following informed written consent. The blood was diluted 1:1 in 0.9% saline and layered over Ficoll Paque (Amersham

Biosciences, Sweden) at a ratio of 3:4 and centrifuged at 800 *g* for 30 minutes. The mononuclear cell layer formed at the interface was aspirated and diluted 1:1 in Hank's balanced salt solution (PAA Laboratories, Austria) before centrifugation at 400 *g* for 10 minutes at 37°C. This procedure was repeated twice to remove residual platelets. Mononuclear cells were resuspended in RPMI 1640, counted in Neubauer hemocytometer (Sigma, USA) and adjusted to the required concentration. Cells were confirmed as 99% viable by the trypan blue exclusion test.

Ferritin labeling of lysosomes. Mononuclear cells (10⁴ cells/ml) in tissue culture tubes (TPP, Switzerland) containing 1 ml of tissue culture medium RPMI 1640 and 40% human serum (PAA Laboratories, Austria) were incubated for 18 hours in CO₂ to promote lysosome formation. The medium was aspirated and cells were further incubated in 1ml RPMI 1640 containing 10 mg/ml ferritin (ICN Biomedicals, Germany) for 3 hours for lysosomal uptake of ferritin.⁵ Cells were rinsed twice with Hank's balanced salt solution to remove free ferritin and then incubated with fresh RPMI 1640 containing 15% human serum for 3 hours to ensure complete labeling of lysosomes. All human serum used in this study were Type AB, off the clot, and purchased from PAA Laboratories, Austria.

Phagocytosis. Opsonization of *B. pseudomallei* was carried out by incubating the organism in 15% human serum for 15 minutes at 37°C. Mononuclear cells (10⁸ cells/ml) were seeded with 10¹⁰ c.f.u /ml of the opsonized organisms and incubated in a water bath at 37°C for 15 minutes.⁶ Phagocytosis was stopped by the addition of chilled RPMI 1640 and the mixture was spun at 800 *g* for 5 minutes at 4°C. This procedure was repeated twice to remove non-phagocyte associated bacteria and the supernatant removed using a sterile pipette. The pellet was suspended in 2 ml of RPMI 1640 supplemented with 15% human serum and reincubated in a water bath at 37°C. At 10, 20, 30, 45, 60 and 120 minutes post infection, 300 µl of the mixture was removed and spun at 800 *g* for 10 minutes. Supernatants were removed and the cell pellets were fixed with 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for a minimum of 4 hours.

Transmission electron microscopy. Following primary fixation in glutaraldehyde, cell pellets were postfixed in 1% osmium tetroxide (Sigma,

USA) for 2 hours at 4°C. Pellets were then rinsed in two changes of 0.1 M cacodylate buffer and dehydrated in graded series of alcohol (50 to 100%), incubated with propylene oxide for 15 minutes and then infiltrated with equal volumes of propylene oxide and resin for one hour. The propylene oxide-resin mixture was replaced with fresh propylene oxide and resin at a ratio of 1:3 and incubated for 2 hours. Pellets were finally embedded in pure resin and allowed to polymerize for 18 hours at 60°C in polyethylene capsules. Polymerized blocks were trimmed, semithin sections were made and mounted on clean glass slides. These were stained with toluidine blue, left to dry on a hot plate and then viewed under a light microscope. Relevant areas were marked and cut using an ultramicrotome (75-85 nm) and these were mounted on clean copper grids. Sections were double stained with uranyl acetate and lead citrate and viewed in a Philips CM12 transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Phagocytosis is a complicated rearrangement of the actin cytoskeleton that delivers extracellular particles into intracellular vacuoles called phagosomes.⁷ The human macrophages formed pseudopodia (Fig. 1) on contact with opsonized *B. pseudomallei* and these pseudopodia then caused the bacilli to be internalized within membrane-bound vacuoles or phagosomes (Fig. 2) probably following depolymerization of the actin microfilaments.⁸ An irregular electron-transparent zone separated each organism from the phagosomal membrane (Fig. 2). Twenty minutes after enclosure of *B. pseudomallei* within a phagosome, fusion with lysosome ensued (Fig. 3) usually leading to the discharge of lysosomal enzymes and other granular constituents into the phagosome. Lysosomes tagged with ferritin, an electron-opaque marker provided evidence of fusion having occurred where clusters of dark ferritin particles were present within the phagosomal compartment (Fig. 3).

Armstrong and Hart^{9,5} working with the facultative intracellular bacteria *Mycobacterium tuberculosis*, reported a set of criteria for classifying bacteria either as damaged or intact, based on their ultrastructural features seen within a macrophage; intact bacteria were seen with (a) low electron opaque central nuclear region, surrounded by; (b) dense bacterial cytoplasm rich in ribosomes and bounded externally by, (c)

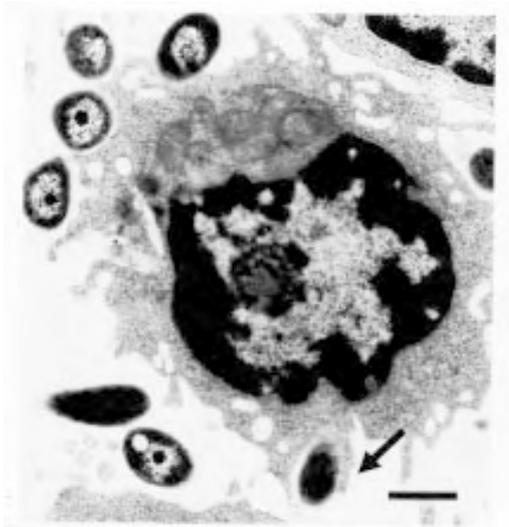


FIG. 1. Pseudopodia embracing *B. pseudomallei* (arrowed). (Bar= 0.8 μ m)

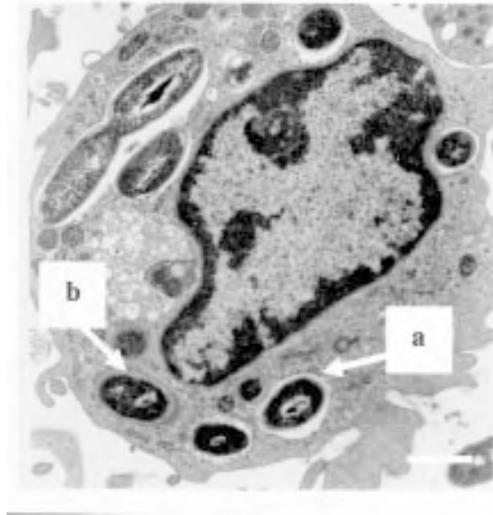


FIG. 2. Internalized *B. pseudomallei* within membrane-bound phagosomes (a). Bacterium seen free within the cytoplasm (b). (Bar= 1 μ m)

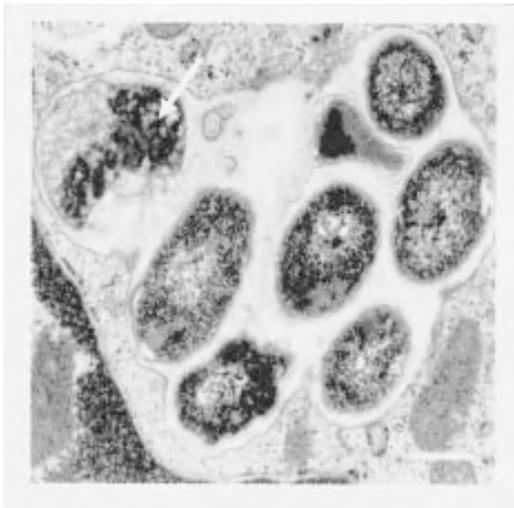


FIG. 3. Fusion (20 min post infection) of ferritin labeled lysosome (arrowed) with a phagosome containing *B. pseudomallei*. (Bar= 0.4 μ m)

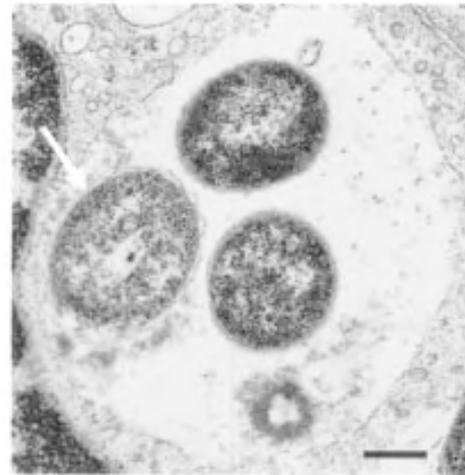


FIG. 4. Damaged *B. pseudomallei* (arrowed) with cavitation, probable separation of cell wall and reduced density of cytoplasm. (Bar= 0.4 μ m)

bacterial plasma membrane and cell wall complex. Damaged bacteria were seen with: (a) gross cavitation affecting the central nuclear region, (b) herniation of cytoplasmic content through breaks in the plasma membrane, (c) lamination often leading to myelin figure formation, involving the bacterial cell wall and its surrounding electron transparent zone, (d) combination of these abnormalities. Using the above criteria, intact bacteria are seen in Fig. 1 and Fig. 2. Damaged bacteria with cavitation of

the central nuclear region is seen in Fig. 4. Degrading bacteria seen at 30–45 minutes post infection showing reduced density of the cytoplasm and “ruffling” of bacterial cell wall (Fig 5). The appearance of ruffling of cell wall may be due to an artifact or perhaps a sign of degradation. Herniation of cytoplasmic contents is seen in Fig. 6. This is unlikely to be a dividing cell as there is no visible cell wall surrounding the protruding cytoplasm. Also dividing cells are more elongated as shown in Figure 2.

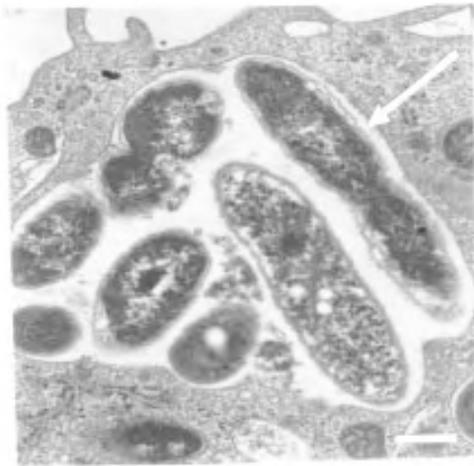


FIG. 5. "Ruffling" of bacterial cell wall (arrowed). (Bar= 0.5µm)

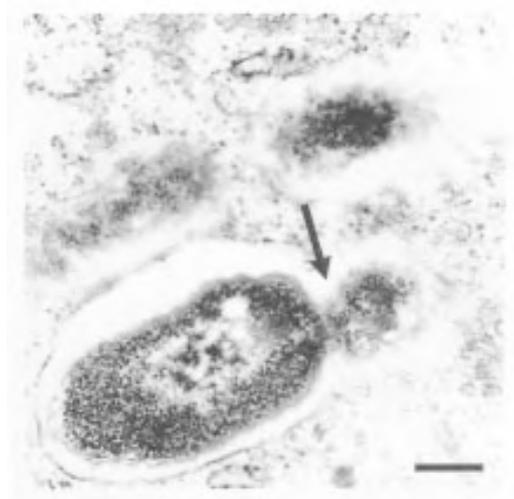


FIG. 6. Break in plasma membrane resulting in herniation of cytoplasmic content (arrowed). (Bar= 1.6µm)

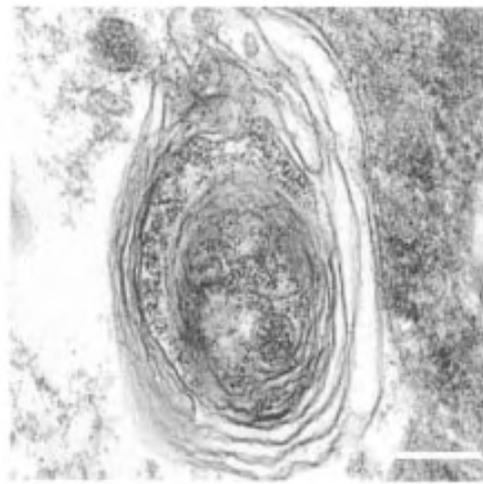


FIG. 7. Lamination of *B. pseudomallei* due to myelin figure formation. (Scale= 200nm)

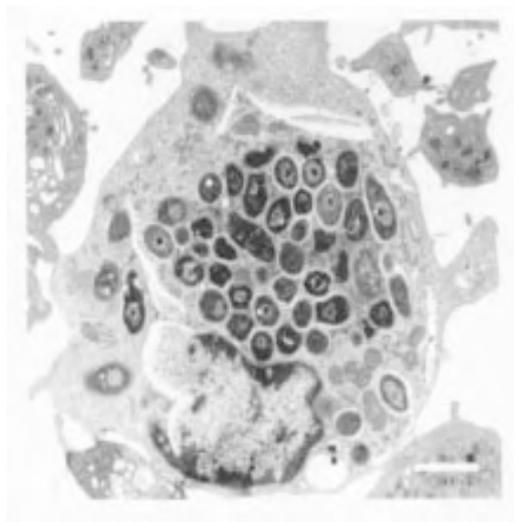


FIG. 8. Intracellular proliferation of surviving *B. pseudomallei* within a macrophage. (Bar= 1.6µm)

Increasing degree of bacillary damage, with myelin figure development is depicted in Fig.7. Mitochondrial damage by ultra fine particles results in the formation of concentric structures known as myelin figures. These structures result from the disassociation of lipoproteins.¹⁰ This phenomenon has not been previously described in *B. pseudomallei*. We observed numerous bacilli packed within the phagosome at 120 minutes post infection indicating proliferation of the surviving bacteria (Fig.8). These large numbers were not due to continued uptake of bacteria as phagocytosis was stopped after 15

minutes. Rupture of the host cell then occurred with the release of *B. pseudomallei* (Fig.9). Although phagosome-lysosome fusion occurred within 20 minutes of post infection and damaged bacteria were seen, fusion and its sequelae failed to ensure complete clearance of all the ingested organisms. The surviving organisms were seen within phagosomes as well as in the cytoplasm i.e. outside the phagosome. A possible explanation for the survival of some of these bacteria maybe their ability to escape from the membrane-bound phagosome into the cytoplasm (Fig. 2). This observation is in accordance with

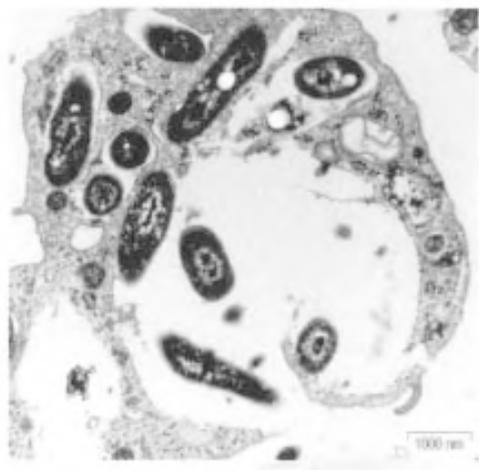


FIG. 9. Ruptured macrophage releasing bacilli into the extracellular milieu.

previous *in vitro* studies demonstrating invasion of *B. pseudomallei* in cell lines where partial or complete dissolution of phagosomal membrane was seen after as little as 15 minutes, probably due to a bacteria-mediated process.⁴ A similar mode of escape into the cytoplasm has been reported in *Listeria monocytogenes*.¹¹ The ability of intracellular pathogens to enter the cytoplasm enables them to reach an environment favourable for survival and growth where nutrients are freely available and microbicidal mechanism(s) do not operate efficiently,¹² hence facilitating intramacrophagal persistence and replication. This may be one of the survival strategies developed by *B. pseudomallei* to remain viable intracellularly.

In conclusion, we are aware of shortcomings in this study such as using only macrophages from one donor, not using a control organism and the appearance of artifacts in the electron-micrographs due to the side effect of shrinkage during dehydration and embedding. We hope to address these issues in our future work.

ACKNOWLEDGMENTS

This study was funded by IRPA Grant 06-02-03-0171 from the Ministry of Science and Technology, Malaysia. (Medical Ethics Committee ref. no. 260.1; 14 August, 2002). We are deeply grateful to all staff of the Electron Microscopy Unit (UM and IMR) for valuable technical assistance. Part of this study was presented as a poster at the 4th Asean Microscopy Conference, 5-6 January, Hanoi, Vietnam and at

the 8th Asia-Pacific Conference on Electron Microscopy, 7-11 June, Kanazawa, Japan

REFERENCES

1. Puthucheary SD, Vadivelu J. Human Melioidosis. Republic of Singapore: Singapore University Press, 2002.
2. Chaowagul W, Suputtamongkol Y, Dance DAB, Rajchanuvong A, Pattara-Arechachai J, White NJ. Relapse in melioidosis: incidence and risk factors. *J Infect Dis* 1993; 168: 1181-5.
3. Jones AL, Beveridge TJ, Woods DE. Intracellular survival of *Burkholderia pseudomallei*. *Infect Immun* 1996 64: 782-90.
4. Harley VS, Dance DAB, Drasar BS, Tovey G. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. *Microbios* 1998; 96: 71-93.
5. Armstrong JA, Hart PD. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. *J Exp Med* 1975; 142: 1-17.
6. Pruksachartvuthi S, Aswapokee N, Thankerngpol K. Survival of *Pseudomonas pseudomallei* in human phagocytes. *J Med Microbiol* 1990; 31: 109-14.
7. Swanson JA, Johnson MT, Beningo K, Post P, Mooseker M, Araki N. A contractile activity that closes phagosomes in macrophages. *J Cell Sci* 1999; 112: 307-16.
8. Bengston T, Jaconi ME, Gustafson M, Magnusson KE, Theler JM, Lew DP, Stendahl O. Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. *Eur J Cell Biol* 1993; 62: 49-58.
9. Armstrong JA, Hart PD. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J Exp Med* 1971; 134: 713-40.
10. Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J, Nel A. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environm Hlth Persp* 2003; 111: 455-60.
11. Ouadrhiri Y, Scoreaux B, Sibille Y, Tulkens PM.. Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by interferon-gamma. *Antimicrob Agents Chemother* 1999; 43: 1242-51.
12. Densen P, Mandell GL. Phagocyte strategy vs. microbial tactics. *Rev Infect Dis* 1999; 2: 817-34.