Comparison by electron microscopy of intracellular events and survival of *Burkholderia pseudomallei* in monocytes from normal subjects and patients with melioidosis

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ABSTRACT

Introduction: ***Burkholderia pseudomallei (B. pseudomallei)*** **has been shown to persist intracellularly in patients with melioidosis, until reactivated by decreasing immunocompetence. We have previously demonstrated by transmission electron microscopy, the internalisation of *B. pseudomallei* by human macrophages and the occurrence of phagosome-lysosome fusion.**

Methods: **Phagocytosis and electron microscopy were used to compare the rate of phagosome-lysosome fusion and the intracellular survival of *B. pseudomallei* using monocytes obtained from five patients with melioidosis and five normal healthy adults.**

Results: **Ingested bacilli were seen in various stages of degradation, with a few remaining viable within phagolysosomes, and the proliferation of these viable bacteria was observed. Phagocytosis of *B. pseudomallei* by normal macrophages was two-fold higher than uptake by the melioidosis macrophages (p-value is less than 0.001). Three times more phagolysosomes were present in the normal macrophages, indicating that fusion occurred slowly and inefficiently in the melioidosis macrophages (p-value is less than 0.001), resulting in higher number of organisms within the melioidosis macrophages (p-value is less than 0.001). Both variables were inversely related to each other.**

Conclusion: **Our observations suggest that phagolysosome fusion occurred slowly and inefficiently in monocytes of patients with melioidosis, leading to an increased number of intracellular organisms compared to monocytes obtained from healthy donors.**

Keywords: ***Burkholderia pseudomallei*****, lysosome, macrophages, melioidosis, phagosome**

Singapore Med J 2006; 47(8):697-703

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INTRODUCTION

Burkholderia pseudomallei (B. pseudomallei), a saprophytic soil bacterium is responsible for melioidosis in humans and animals. The organism is widely distributed in Southeast Asia but there are certain geographical areas of high endemicity in this region. Infection by this gram-negative organism may cause septicaemic or non-septicaemic melioidosis. The acute septicaemic form may affect various organs throughout the body and carries a high mortality rate of over 40%[1].

A remarkable feature of the infection is its capacity for latency, relapse and recurrence, especially in immunocompromised hosts, and this can occur in spite of appropriate and prolonged antimicrobial therapy. Such infections have been assumed to be the result of failure by the host to eliminate the initial organism of the primary infection. Molecular techniques such as ribotyping and pulsed field gel electrophoresis (PFGE) have shown that repeated episodes of infections in melioidosis patients are due to the original infecting strain[2]. Many factors are thought to be responsible for this but the most important is that *B. pseudomallei* occupies an intracellular niche, and intracellular replication and localisation of the bacterium have been documented in-vivo[3,4] as well as in-vitro in both phagocytic and non-phagocytic cell lines[5,6].

The exact mechanism of this intracellular survival is not well understood but it must undoubtedly involve complex interactions between the microorganism and the host's ability to limit infection. It is believed that intracellular killing does not occur efficiently in certain immunocompromised hosts and in patients with severe resistance-lowering basic diseases such as diabetes mellitus or hepatic cirrhosis, an intact cell-mediated immune mechanism is vital in controlling this intracellular pathogen[7]. Intracellular pathogens have developed strategies to evade host defense mechanisms, such as inhibition of phagosome-lysosome fusion, phagosomal acidification, resistance to lysosomal contents and cationic peptides, and interference with reactive oxygen and nitrogen intermediates[8].

The interaction of the host cell and *B. pseudomallei* has been extensively studied using mouse macrophage cell line RAW 264.7, Hela cells and U937 human macrophage-like cell lines. In the present study, we used human mononuclear cells obtained from healthy donors and melioidosis patients to show by transmission electron microscopy that *B. pseudomallei* is able to invade and survive intracellularly in human macrophages and to identify possible mechanisms by which the organism may overcome the host defenses.

METHODS

An arabinose-negative strain of *B. pseudomallei* was isolated from a septicaemic patient on antibiotic therapy at the University of Malaya Medical Centre, using routine bacteriological methods and confirmed by the API 20NE system (bioMerieux, Marcy-Etoile, France) and agglutination with specific monoclonal antibody (All Eights, Kuala Lumpur, Malaysia). The strain was kept in Brain Heart Infusion Broth (Becton Dickinson, San Jose, USA) containing 15% glycerol at –70°C. The organism was grown on blood agar plates and then in nutrient broth, incubated for 18 hours at 37°C, spun at 400 g for five minutes and the supernatant was removed. The bacterial pellet was then opsonised for 30 minutes at 37°C with RPMI 1640 (PAA Laboratories, Linz, Austria) containing 15% human serum, type AB, off the clot, which was purchased from PAA Laboratories, Linz, Austria.

Heparinised blood was obtained, following informed consent, from five healthy adult donors who were not on any medication, not diabetics and were serologically negative for melioidosis. Similar samples were taken from five septicaemic melioidosis patients who were all diabetics, on antimicrobial therapy but not bacteraemic at the time of bleeding. The isolation of mononuclear cells, ferritin labelling of lysosymes and processing of specimens for electron microscopy were according to protocols published previously[9]. Briefly, mononuclear cells were isolated from the blood samples using Ficoll Paque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). The cell layer formed at the interface was aspirated and diluted 1:1 in Hank's balanced salt solution (PAA Laboratories, Linz, Austria) before centrifugation at 400 g for ten minutes at 37°C. This procedure was repeated twice to remove residual platelets. The cells were resuspended in RPMI 1640, counted in a Neubauer haemocytometer (Sigma, St. Louis, MO, USA), adjusted to the required concentration, and confirmed as 99% viable by the trypan blue exclusion test.

The mononuclear cells (10^4 cells/ml) were further incubated with 40% human serum for 18 hours in CO_2 to promote lysosome formation. Ferritin labelling was carried out by incubating the cells in RPMI 1640 containing 10 mg/ml electron-dense ferritin (ICN Biomedicals, Eschwege, Germany) for a total of six hours[10]. These cells in tissue culture tubes were seeded with opsonised *B. pseudomallei* at a concentration of 10^{10} cfu/ml and incubated in a water bath at 37°C for 15 minutes[3]. Phagocytosis was stopped by the addition of chilled RPMI 1640 and the mixture spun at 800 g for five minutes at 4°C. This procedure was repeated twice to remove non-ingested bacteria from the cells containing ingested bacteria. The pellet was resuspended and re-incubated with 15% human serum at 37°C. At 0, 10, 20, 30, 45, 60 and 120 minutes post-infection, 300 μl of the mixture was removed, spun at 800 g for ten minutes and the cell pellets fixed with 4% glutaraldehyde, postfixed in 1% osmium tetroxide (Sigma-Aldrich, St. Louis, USA) and dehydrated through graded solutions of alcohol. Dehydrated pellets were incubated with propylene oxide and infiltrated with a mixture of propylene oxide and resin for an hour. Pellets were then embedded in pure resin and allowed to polymerise at 60°C for 18 hours.

The polymerised blocks were trimmed, and ultrathin sections (75-85 nm) were made and mounted on copper grids, stained and viewed using a Philips CM12 transmission electron microscope (TEM) at an accelerating voltage of 80 kV. Three sections representing levels throughout each pellet were examined. Ten fields for each section were examined for the number of intact intracellular bacteria and the number of fused ferritin-labelled phagolysosomes, taking precaution not to count the same field twice or more. Damaged bacteria using the criteria set by Armstrong and Hart were excluded from counting[10]. The mean of 30 fields was calculated for each pellet at each time-point and the mean of five was calculated for each time-point for both groups of subjects (Table I).

The mean number of intracellular *B. pseudomallei* and phagosome-lysosome fusions obtained for the five normal and five melioidosis subjects were compared using the independent sample t-test (One-way ANOVA) with the assistance of the Statistical Package for Social Sciences (SPSS) version 6.0 (Chicago, IL, USA). A p-value of <0.001 was considered to be statistically significant.

Table I. Mean number of intracellular *B. pseudomallei* and phagosome-lysosome fusion in monocytes from normal and melioidosis subjects.

	Normal (n=5)		Melioidosis (n=5)	
Time-point (minutes)	Intracellular *B. pseudomallei*	Phagosome-lysosome fusion	Intracellular *B. pseudomallei*	Phagosome-lysosome fusion
10	4.79	1.90	2.86	1.02
20	14.49	4.94	11.98	3.85
30	12.43	6.95	15.04	2.35
45	9.91	9.31	17.40	1.71
60	13.19	5.96	21.46	1.05
120	16.92	2.91	35.62	0.55
Mean	11.95	5.33	17.39	1.75

Table II. Independent sample t-test: comparison of the mean values of intracellular *B. pseudomallei* and phagolysosome fusions.

Time (minutes)	Variables	Subject	Mean	Standard deviation	p-value
10	IcBps	normal	4.79	0.38	<0.001
	melioidosis	2.86	0.28		
	P-ly	normal	1.90	0.21	<0.001
	melioidosis	1.02	0.11		
20	IcBps	normal	14.49	0.69	<0.001
	melioidosis	11.98	0.55		
	P-ly	normal	4.94	0.24	<0.001
	melioidosis	3.85	0.32		
30	IcBps	normal	12.43	0.77	<0.001
	melioidosis	15.04	0.50		
	P-ly	normal	6.95	0.24	<0.001
	melioidosis	2.35	0.22		
45	IcBps	normal	9.91	0.64	<0.001
	melioidosis	17.40	0.62		
	P-ly	normal	9.31	0.46	<0.001
	melioidosis	1.71	0.18		
60	IcBps	normal	13.19	0.41	<0.001
	melioidosis	21.46	1.08		
	P-ly	normal	5.96	0.23	<0.001
	melioidosis	1.05	0.08		
120	IcBps	normal	16.92	0.50	<0.001
	melioidosis	35.62	1.03		
	P-ly	normal	2.91	0.21	<0.001
	melioidosis	0.55	0.09		

IcBps: intracellular *B. pseudomallei*; P-ly: phagosome-lysosome fusion

RESULTS

The macrophages formed pseudopodia and internalised the opsonised *B. pseudomallei* (Fig. 1) into plasma membrane-derived vacuoles or phagosomes within minutes. Inside the phagosome, many intact bacteria, i.e. not damaged, were visible (Fig. 2). They had an overall low electron opaque central nuclear region, high-density bacterial cytoplasm rich in ribosomes and bounded externally by bacterial plasma membrane with an outer cell wall complex[11]. The fusion of phagosome and lysosome occurred within 20 minutes post-infection and was confirmed by the labelling of lysosomes with ferritin, which were recognised as small, highly electron-dense granules (Fig. 3) that were present as clusters within the phagosomal compartment. Inside the phagolysosome, "damaged" bacteria were seen with gross cavitation within the central nuclear region, ruffling of the cell

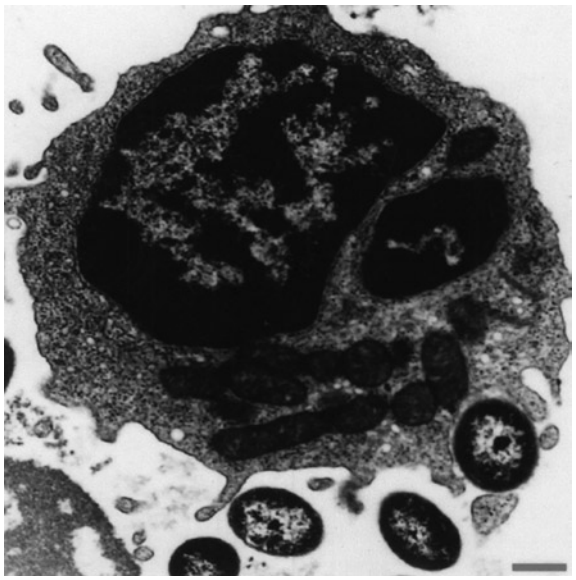

Fig. 1 Pseudopodia embracing *B. pseudomallei* at 0 minute post-infection. (Bar=0.8μm).

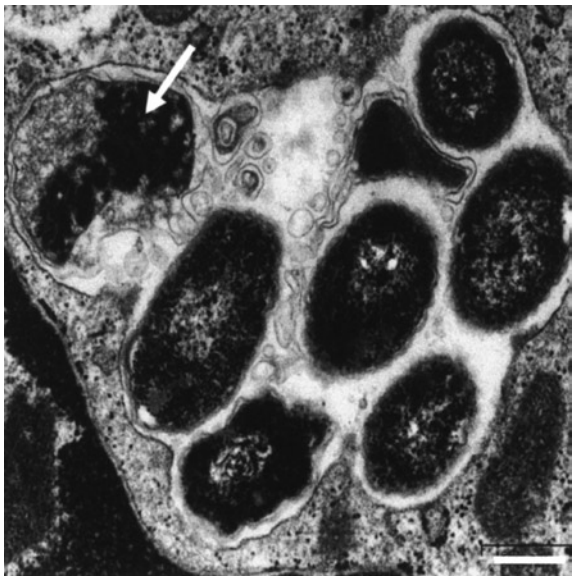

Fig. 3 Fusion (20 minutes post-infection) of ferritin-labelled lysosome (arrowed) with a phagosome containing *B. pseudomallei* (Scale=500 nm). (Reproduced with permission from the College of Pathologists, Academy of Medicine, Malaysia).

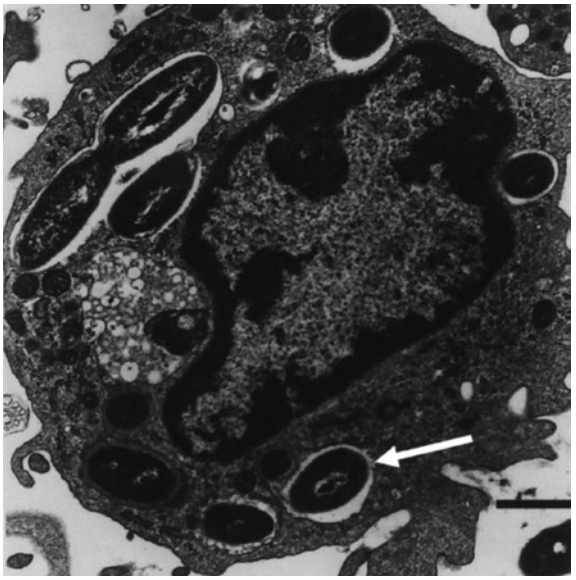

Fig. 2 Internalised *B. pseudomallei* within membrane-bound vacuoles or phagosomes (arrowed), ten minutes post-infection (Bar=1μm). (Reproduced with permission from the College of Pathologists, Academy of Medicine, Malaysia).

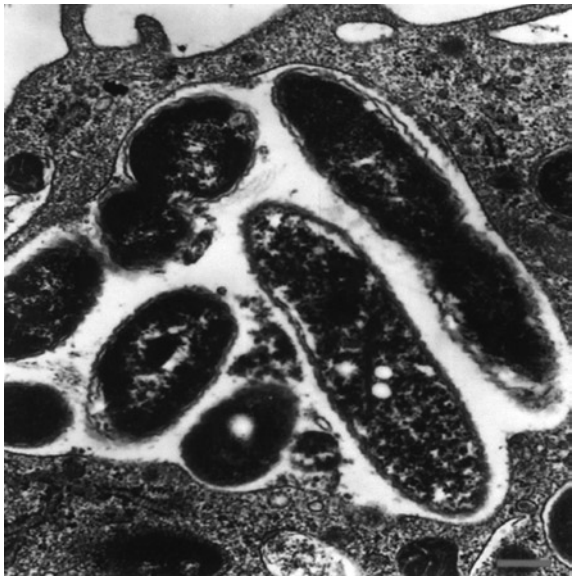

Fig. 4 Ruffling of bacterial cell wall, 30 minutes post-infection (Bar=0.5μm). (Reproduced with permission from the College of Pathologists, Academy of Medicine, Malaysia).

wall (Fig. 4) and breaks in plasma membrane resulting in herniation of cytoplasmic contents.

Numerous intact and damaged bacteria, were present at 60 minutes post-infection (Fig. 5). The intact bacilli were seen to survive and multiply and eventually two hours post-infection, the phagolysosomal membrane ruptured, with the bacteria escaping into the cytoplasm followed by rupture of the macrophage itself, releasing the bacilli into the extracellular milieu (Fig. 6) and probably initiating new cycles of infection. The number of intracellular bacteria and fused phagolysosomes were counted in ten fields for each section at each time-point in infected macrophages obtained from both normal and melioidosis subjects. The average of 30 fields (i.e. three sections) was calculated (Table I) and the mean values, standard deviation and p-values were determined (Table II).

During the initial phase, i.e. 10 to 20 minutes post-infection, more bacilli were phagocytosed by the normal macrophages than by those from the melioidosis patients, indicating a better uptake of the bacteria by the normal macrophages. At similar time-points, there were twice the number of fused

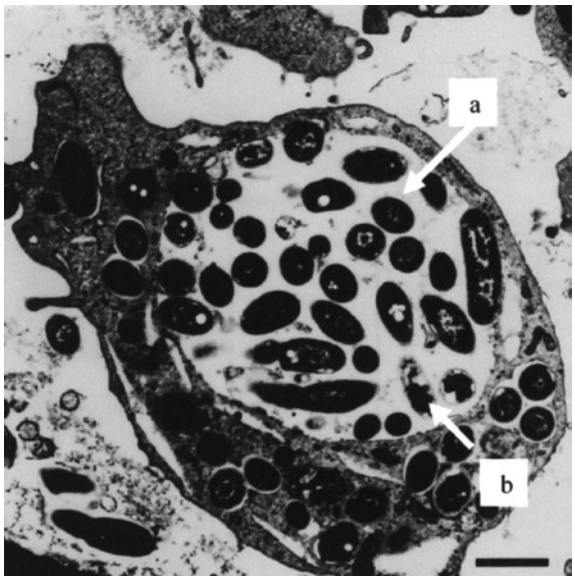

Fig. 5 Proliferation of surviving *B. pseudomallei* within a macrophage, 60 minutes post-infection. Both intact (a) and damaged (b) bacteria are seen. (Bar=1.6μm)

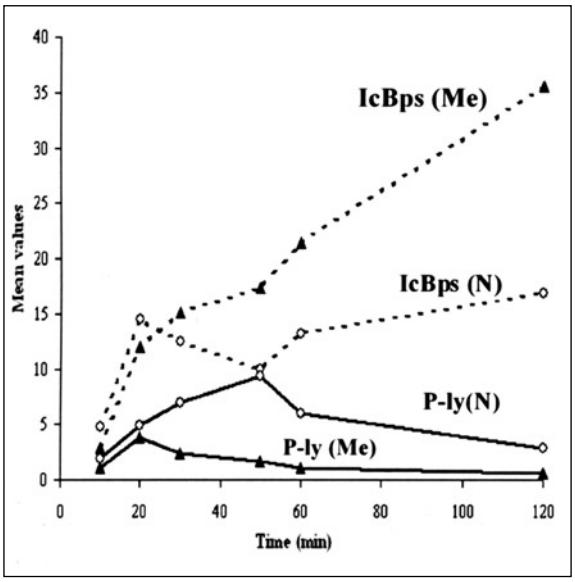

Fig. 7 Dynamics of interaction between intracellular *B. pseudomallei* and phagosome-lysosome fusion. (IcBps: intracellular *B. pseudomallei*; P-ly: phagosome-lysosome fusion; N: normal macrophage; Me: melioidosis macrophage). Standard deviations are given in Table II.

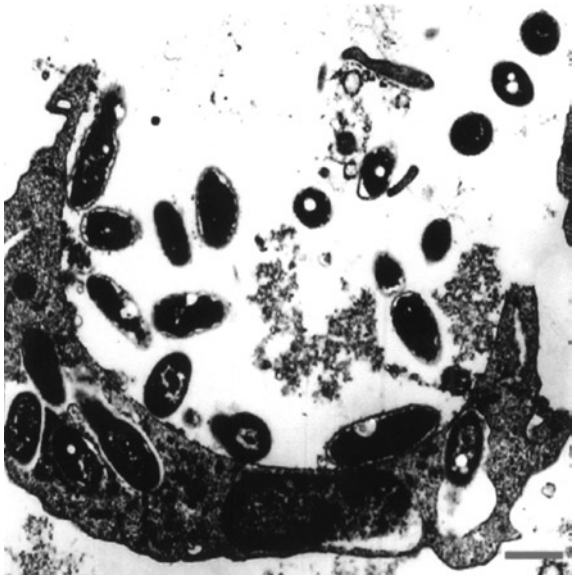

Fig. 6 Ruptured macrophage, 120 minutes post-infection releasing bacilli into the extracellular milieu. (Bar=1.6μm) (Reproduced with permission from the College of Pathologists, Academy of Medicine, Malaysia).

phagolysosomes present in the normal compared to the melioidosis cells. At 30 minutes post-infection, there was a dramatic increase in the number of bacteria in the melioidosis cells compared to the normal cells, but the opposite happened with the phagosome-lysosome fusion which increased in the normal cells but began to decrease in the melioidosis macrophages after peaking at 20 minutes (Fig. 7).

The number of phagosome-lysosome fusions peaked at 45 minutes post-infection in the normal macrophages causing the intracellular bacteria to decrease from 12.4 at 30 minutes to 9.9 at 45 minutes (Table I). In contrast, phagosome-lysosome fusion decreased in the melioidosis cells with a concomitant increase in the intracellular bacteria. Thereafter, the number of fused phagosomes began to decrease in both types of cells, probably due to the limited viability of the macrophages. Subsequently, there was an increase in the intracellular organisms but the number of bacteria was significantly higher in the melioidosis cells than in the normal macrophages ($p<0.001$).

Both variables were inversely related to each other whereby an increase in the number of fused phagolysosomes resulted in a decreasing number of intracellular bacteria, and vice versa (Fig 7). The mean number of *B. pseudomallei* surviving within melioidosis macrophages was 17.39 which was significantly higher than that of the normal macrophages which showed a mean of 11.95 ($p<0.001$). The occurrence of phagosome-lysosome fusion similarly showed a mean of 5.33 and 1.75 for the normal and melioidosis macrophages, respectively ($p<0.001$).

DISCUSSION

The process of engulfing a foreign particle, i.e. phagocytosis, is of fundamental importance for a wide variety of organisms. For the host, this process either limits or resolves the infection, but for the pathogen, it is either an opportunity or an obstacle to replication. Phagocytosis is a membrane-directed process driven by the host cell actin cytoskeleton that results in internalisation of particles the size of bacteria and yeasts[12]. However, pathogens are able to survive

within phagocytes by various mechanisms, such as by remodelling their phagosomes (*Mycobacteria*), by moving out of the phagosomes (*Listeria*), by actively interfering with or antagonising phagosome-lysosome fusion[13]. or by resisting the hostile environment of the mature phagosome (*Coxiella*)[14].

By the quantitative approach in this study, we have shown that the occurrence of phagosome-lysosome fusion was significantly lower in the *B. pseudomallei*-infected melioidosis macrophages than in those obtained from normal subjects, leading to a significantly higher number of organisms surviving within the monocytes from the melioidosis subjects (Fig. 7). Our observations indicate that the phagosome-lysosome fusion mechanism in *B. pseudomallei*-infected macrophages and the subsequent metabolic oxidative burst failed to ensure complete clearance of the organism, thus allowing intracellular survival and proliferation which probably gives rise to relapse and recurrence in patients with melioidosis.

This low incidence of phagosome-lysosome fusion may be due to many factors present in the organism and/or the host cells that may act singly or in concert to prevent this fusion. One can only speculate that the biochemical nature of either the capsule or the cell wall of *B. pseudomallei* may influence the interaction of the phagosomal wall with the lysosome. The death or survival of ingested bacteria is related to the metabolic events in the respiratory burst following phagocytosis and the normal oxidative function of the phagocyte must be intact for this to occur. The presence of numerous intact bacteria at 60 minutes post-infection may be due to the fact that *B. pseudomallei* was either able to resist killing by the oxidative burst or perhaps suppress the continuous process of the oxidative metabolic burst. The lipopolysaccharide (LPS) of *B. pseudomallei* has an unusual acid-stable structure in its inner core region attached to the lipid A moiety. This unique structure may affect the binding affinity of LPS with the cell receptors, which could result in its weaker macrophage-activating activity[15].

We have shown, in a previous study[16], that *B. pseudomallei*-infected macrophages from melioidosis patients produced significantly lower levels of nitric oxide than macrophages from normal subjects. Nitric oxide is one of the reactive nitrogen intermediates which is a major microbicidal mechanism in phagocytic cells. The reduced efficiency of this oxygen-dependant mechanism may be one of the factors responsible for the non-elimination of the organisms from *B. pseudomallei*-infected macrophages. In the same study, we also postulated that low levels of 8-iso-$PGF_{2\alpha}$ produced in the melioidosis macrophages indicate inefficient activation of free radical production leading to incomplete elimination of the intracellular organism.

Type III secretion systems found on pathogenicity islands confer major virulence traits on some gram-negative intracellular pathogens, and such a system has been identified in *B. pseudomallei*[17]. A subset of type III secretion secreted proteins (translocators) is believed to interact with the eukaryotic cell membrane and mediate the delivery of secreted effector proteins. Once inside host cells, the effector proteins subvert host cell processes to the benefit of the bacteria[18]. This probably enables the organism to survive and multiply unrestricted in an environment where nutrients are freely available and the microbicidal system does not operate efficiently[19]. Stevens et al have also reported that mutant strains of *B. pseudomallei* devoid of this secretion apparatus were unable to escape from the endocytic vacuole and exhibited impaired intracellular survival. Therefore, the type III secretion system might be instrumental in mediating the escape of *B. pseudomallei* from the phagosome into the host cytoplasm[18].

We have demonstrated that *B. pseudomallei* are able to invade and survive intracellularly in human macrophages. Phagosome-lysosome fusion after invasion did occur but slowly and inefficiently in monocytes obtained from the melioidosis patients. Although the number of intracellular organisms decreased with the increase in phagosome-lysosome fusions, ultimately, the number of *B. pseudomallei* dramatically increased with the reduction in the number of fused phagolysosomes in the melioidosis monocytes. Taken together, our observations suggest that a small number of *B. pseudomallei* are able to overcome the microbicidal armamentarium of the human host cell, to persist and multiply or perhaps remain latent in a dormant state, only to multiply at a later date, giving rise to relapse and recurrence of melioidosis.

In conclusion, we are aware of shortcomings in this study, such as not using a control organism and the appearance of artifacts in the electronmicrographs due to the side effect of shrinkage during dehydration and embedding. Future work should address these factors as well as use a larger number of human subjects to verify our findings.

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 (University of Malaya and Institute for Medical Research, Kuala Lumpur) for their valuable technical assistance.

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