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ORIGINAL ARTICLE

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Atypical morphological characteristics and surface antigen expression of *Burkholderia pseudomallei* in naturally infected human synovial tissues

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Abstract *Burkholderia pseudomallei* is the causative agent of melioidosis, a disease that frequently runs a protracted course and is very difficult to eradicate. The mechanisms that this organism uses to escape from host defense mechanisms and antibiotics are not well understood. The aim of the study was to compare the morphological characteristics and surface antigen expression of *B. pseudomallei* in naturally infected human synovial tissues with the characteristics of bacteria grown in culture media. Immunoelectron microscopic study was performed in four synovial biopsies taken from four *B. pseudomallei* septic arthritis patients. Colonies of pathogenic *B. pseudomallei* collected from culture media were used as positive controls. Polyclonal antibody to whole cell *B. pseudomallei* was used as a primary antibody. Complete bacteria-like particles were demonstrated both extracellularly and intracellularly in all four synovial specimens. The intracytoplasmic location of *B. pseudomallei* and mononuclear phagosome containing microcolony-like structures were demonstrated. *B. pseudomallei* found in the synovial membrane samples were mostly atypical, with fewer cytoplasmic electron lucent granules. Immunogold staining of bacterial surface antigens was weaker than staining of positive controls. We demonstrated atypical forms of *B. pseudomallei* and evidence for suppression of its surface antigens in naturally infected human synovial tissues. This adaptation may help bacteria to survive despite host immune surveillance and treatment with antibiotics.

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Introduction

Burkholderia pseudomallei, a facultative intracellular gram-negative bacillus, is a causative agent of melioidosis, a serious infection that is endemic primarily in Southeast Asia and in tropical Australia. Melioidosis in Western countries is extremely rare. It occurs only in those who returned from endemic areas. The presenting symptoms usually appear within a few weeks, but can be delayed as long as 18 years after exposure to this organism.[1] Thus, diagnosis of melioidosis in Western countries is usually difficult and delayed because lack of familiarity with this infection. A high index of suspicion is fundamental for early recognition of this disease, particularly in febrile patients who have recently returned from endemic areas. Clinical manifestations of melioidosis range from subclinical to acute localized forms, acute septicemic, and chronic forms.[2] Infection with this organism occurs more frequently in patients with underlying diseases, particularly diabetes and chronic renal failure.[3]

Even though much has been learned about the pathogenesis of melioidosis, the difficulty of eradicating the infection and its protracted course are still problematic. 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, should raise concerns during treatment of this infection. Recent studies indicated that relapses of melioidosis were the result of reactivation of a persistent endogenous source of infection.[4–6]

Many studies have suggested that the survival of *B. pseudomallei* in host tissue is caused by the intracellular location of *B. pseudomallei* and its ability to suppress host defense mechanisms.[7–10] Studies have demonstrated that *B. pseudomallei* can inhibit chemotaxis and cellular oxidative

burst,[7,11] and resist some specific cellular processes in the bactericidal activity of phagocytic cells, e.g., cationic peptides and human defensin.[12] These survival mechanisms in host tissue may be related to the polysaccharide capsules of the organisms.[13–16]

Not only is the infection caused by *B. pseudomallei* very difficult to treat, but a similar difficulty in eradicating infection can occur with other intracellular organisms such as *Chlamydia trachomatis*, one of the causative agents of reactive arthritis (ReA) and Reiter's syndrome. Even though molecular evidence supports the viability of *C. trachomatis* in the synovial tissue,[17] it is still very difficult to cure this infection with long-term antibiotics.[18] Our previous immunoelectron microscopic (IEM) work on such patients demonstrated the morphological transformation and lower surface antigen expression of *C. trachomatis* in synovial membrane samples.[19] This finding supported the earlier study of Beatty et al.[20] After they exposed *C. trachomatis* to IFN-γ, transformation of bacteria and reduction of surface antigen expression were demonstrated. They also found that the effect of IFN-γ on the Chlamydial developmental cycle was dose-dependent. From our previous IEM study, we postulated that the biological adaptation of *C. trachomatis* may contribute to its persistence in synovial tissue, and may cause chronic long-standing arthritis in these patients. Whether a similar biological change is a possible in the pathogenesis of *B. pseudomallei* infection has never been studied.

We designed an IEM study to define the ultrastructural morphology of *B. pseudomallei* and its surface antigen expression in naturally infected synovial tissue. This may expand our understanding of bacterial survival mechanisms in host tissues, and may lead to an innovative idea about how this infection could be treated effectively.

Patients and methods

Patients

Clinical data. Four *Burkholderia pseudomallei* septic arthritis patients were studied. Their clinical data and evidence from the bacteriological studies are summarized in Table 1.

Synovial biopsies. Synovial biopsies were done during arthroscopic drainage or arthrotomy at the affected joints.

Methods

Tissue preparation for immunoelectron microscopy. All synovial membrane samples, size 1 mm^3, were immediately fixed for 1 h in 2.5% glutaraldehyde, thoroughly washed in 0.1 M cacodylate buffer, dehydrated in graded ethanol, and embedded in Epon 812. After light-microscopic screening on semithin sections, ultrathin sections were cut from selected areas, transferred to nickel mesh grids, and then dried overnight.

Positive control. Pathogenic *B. pseudomallei* colonies were used as positive controls. Tissue fixation and procession were identical to the procedures performed in the patients' synovial membrane samples described above.

Immunogold staining. The postembedding immunogold staining (IGS) technique for *B. pseudomallei* antigen detection was initially developed on the positive controls using 10-nm gold conjugates. Rabbit polyclonal antibodies to whole cell *B. pseudomallei* (pooled) Y571 II RS (kindly provided by Chaowakul V, Medical Department, Sumprasithiprasong General Hospital, Ubonrajathani, Thailand) was used as a primary antibody. This primary antibody primarily react against lipopolysaccharide (LPS) of *B. pseudomallei*. The antibody was diluted in 2% bovine serum albumin in phosphate buffered saline buffer (2% BSA–PBS) (pH 7.4) in various concentrations. Optimal immunogold staining was reached at a 1:1500 dilution. Immunogold conjugated goat antirabbit antibody (10 nm) was used as a secondary antibody. The antibody was diluted 1:30 with 2% BSA–PBS (pH 7.4).

Immunostaining procedure. The grids were rinsed for 10 min on the surface of distilled water in the wells of microtest plates, and then incubated, as a blocking step,

Table 1. Clinical data from four *B. pseudomallei* septic arthritis patients

Patient #	Underlying disease	1st source of infection	Site of arthritis / onset	Culture results at the onset of arthritis	Antibiotics given before synovial biopsy was performed
1 DV	Gout	Acute right ankle arthritis H/C & ankle SF +ve	Right knee / acute	SF & syn culture −ve Syn PCR +ve	Ceftazidime + cotrim. × 2 weeks
2 CH	DM	Acute pyomyositis, right leg Pus C/S +ve	Right knee / acute	SF +ve, H/C +ve	Doxycycline + cotrim. × 18 days
3 PT	None	Subacute pulmonary infection	Left elbow / acute	SF +ve	No
4 PN	DM	Chronic pulmonary infection	Right shoulder / subacute	SF +ve, H/C +ve	Ceftazidime + cotrim. × 1 week

SF, synovial fluid; syn, synovial membrane; cotrim, cotrimoxazole; PCR, polymerase chain reaction for *B. pseudomallei*; H/C, hemoculture

with 5% BSA–PBS (pH 7.4) for 20 min at room temperature. After the excess serum was blotted off, grids were incubated with the primary antibody for 1 h at room temperature, and then gently washed three times, for 5 min each time, on a shaking table with 1% gelatin–PBS (pH 7.4). Excess buffer was blotted off and the grids were incubated with secondary antibody for 1 h at room temperature. Finally, the grids were washed with 1% gelatin–PBS (pH 7.4) twice for 5 min each time, washed with distilled water twice for 5 min each time, and then dried. The grids were stained with 2.5% uranyl acetate for 6 min prior to examination using a Zeiss EM-10 electron microscope under a 100-kV beam.

Negative controls. Negative controls were produced by omitting the primary antibodies, using monoclonal antibody to Salmonella LPS as the primary antibody, and using an irrelevant secondary antibody.

Results

Clinical data (Table 1)

Out of four patients studied, three had concomitant diseases, and all had a primary source of infection. In three patients (patients 1, 2, and 4) the arthritis occurred during antibiotic treatment, from 1 week to almost 3 weeks. Bacteriological diagnosis was confirmed by positive synovial fluid culture of *Burkholderia pseudomallei* in three patients (patients 2, 3, and 4), including two of the patients who were being treated with antibiotics (patients 2 and 4).

In one patient whose bacteriological diagnosis was not determined by positive culture (patient 1), acute knee arthritis occurred 2 weeks after treatment of a *B. pseudomallei* septic ankle. This was a few days after parenteral antibiotics had been switched to an oral form when the septic ankle was considered to have subsided. Colchicine was started initially since the synovial fluid analysis revealed intracellular urate crystals. As the patient's inflamed knee became progressively worse, arthroscopic drainage and synovial biopsy were performed. Histopathological examination of the synovial membrane showed suppurative synovitis. Synovial membrane culture was negative, but positive polymerase chain reaction (PCR) for *B. pseudomallei* confirmed the diagnosis. The diagnosis of a *B. pseudomallei* septic knee was eventually further supported by the dramatic clinical response to readministration of parenteral antibiotics.

All patients clinically improved after surgical drainage and 2–4 weeks parenteral antibiotic treatment. The antibiotics were continued in oral form for all patients for 20 weeks, as is the standard recommendation.

IEM findings of positive control

The ultrastructural characteristics *of B. pseudomallei* grown in culture media are shown in Fig. 1. Immunogold staining (IGS) to whole cell *B. pseudomallei* antigens localized rather specifically and densely along the bacterial envelope. Atypical forms of *B. pseudomallei* were rarely found, and IGS on these types of organism was varied among particles, as shown in Fig. 2.

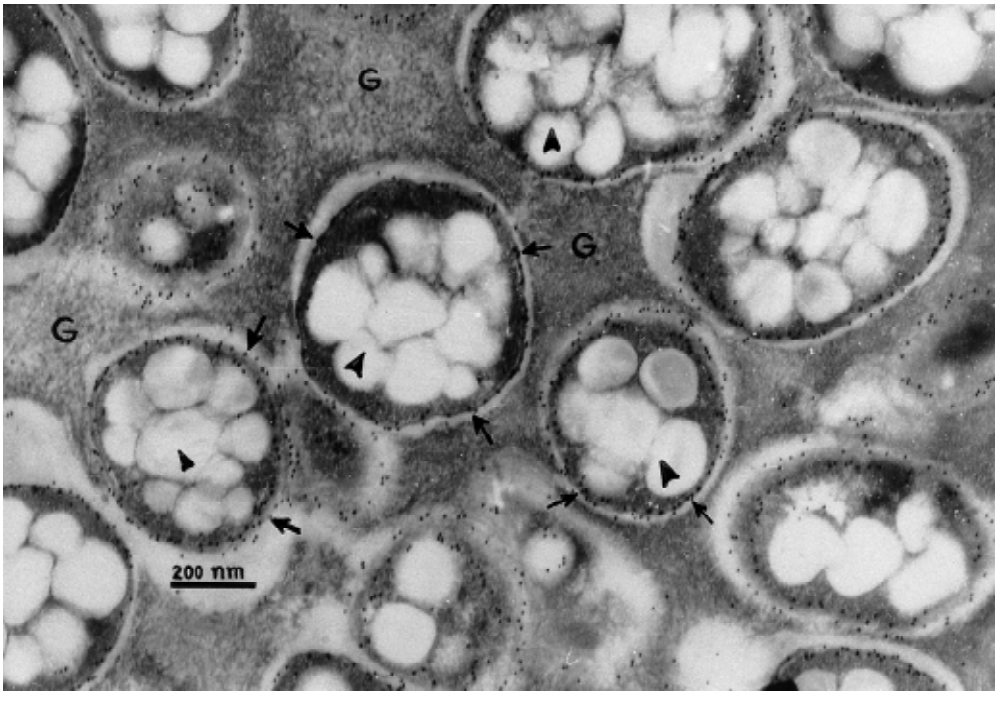

Fig. 1. Ultrastructural characteristic of *B. pseudomallei* in culture media. A colony of *B. pseudomallei*. Large electronlucent granules (*arrow heads*) within the bacterial cytoplasm probably represents poly-β-hydroxybutyrate accumulation, which was described as an ultrastructural characteristic for *Burkholderia* species. Electron-dense intercellular materials (*G*) probably represent the bacterial capsule (glycocalyx). Strong surface antigen expression is indicated by dense immunogold staining (IGS) along the bacterial envelope (*arrows*)

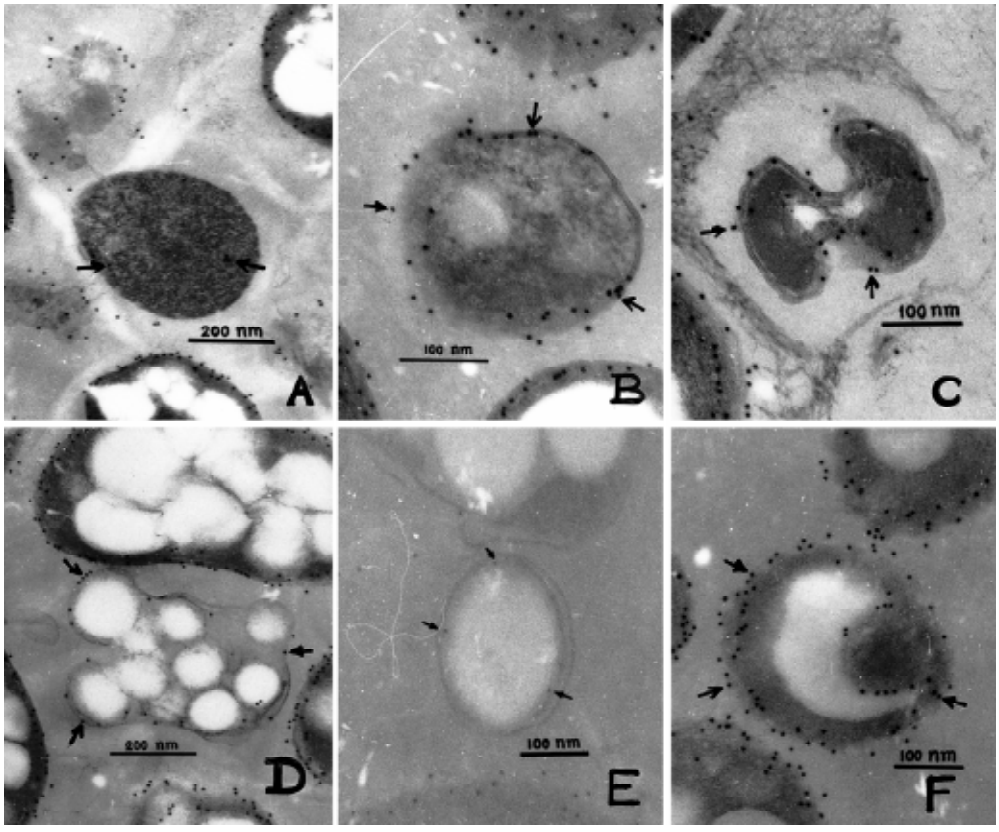

Fig. 2. Atypical ultrastructural morphology of *B. pseudomallei*. These atypical forms were rarely seen in the positive controls. **A–C** Absence of electron-lucent granules; **D**, **E** thin membrane-coated isolated electron-lucent granules; **F** bull's eye appearance. Variations in surface antigen expression was shown by immunogold staining (*arrows*)

Table 2. Ultrastructural findings in four synovial samples of *B. pseudomallii* septic arthritis

Patient #	Inflammatory cells Infiltration	Phagocytic activity	Bacteria				
			Extracellular bacteria	Intraphagosome		Intracytoplasmic	Microcolony
				Complete	Residual		
1 DV	+++ Mono, scant PMN	++	+	+	+	+	+
2 CH	++ to +++[a] Mono / lymph, few PMN & mast cells, perivascular infiltration	+	+				+
3 PT	++++ Mono, few PMN	++++	+	+	+++	+	+
4 PN	Fibroblast +++,[b] mono, few PMN, perivascular infiltration	+	+		+ In endothelial cell		

Mono, mononuclear cells; PMN, polymorphonuclear cells
[a] Patient 2. PMN found close to mononuclear cells containing bacterial microcolonies. There was vascular injury with bacterial debris found in endothelial cells and the perivascular area
[b] Patient 4. Deep synovial tissue contained proteinaceous exudate; endothelial cells showed some phagocytic activity

IEM findings of synovial membranes

The ultrastructural findings for all four synovial specimens are summarized in Table 2.

The histological findings in all four synovial tissue samples were basically synovial proliferation with prominent mononuclear cell infiltration. There was little PMN infiltration even in the early-onset case (patient 3). The amount of proteinaceous and cellular exudate and phagocytic activity noted in the infected synovial tissues did not seem to be related to the onset of disease. Vascular injury and endothelial phagocytic activity were also noted in a patient whose arthritis developed during antibiotic treatment and corresponded with his positive hemoculture

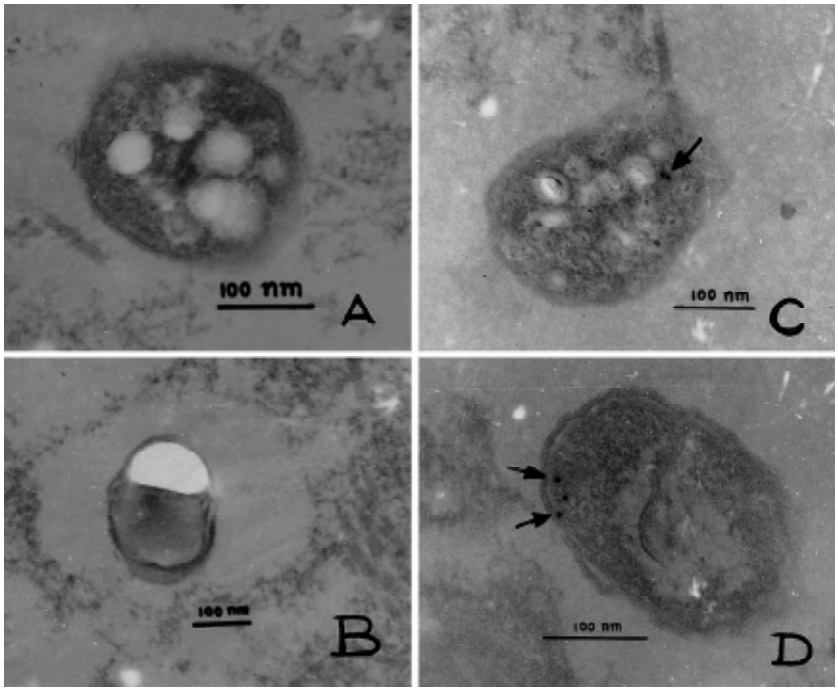

Fig. 3. Extracellular bacteria-like structures in naturally infected synovium. **A** Ultrastructural characteristics of *B. pseudomallei*, a membrane-bounded bacterium containing large electron dense granules within the cytoplasm, was shown in only one patient (patient 3). Surface antigen expression on this bacterium was totally absent, which was significantly different from the abundant surface antigen expression of *B. pseudomallei* grown in culture media and shown in Fig. 1. **B–D** Most extracellular bacteria found in our patients were atypical. **B** Membrane-bounded isolated electron-lucent granule, and **C**, **D** membrane-bounded bacteria without intracytoplasmic electron-lucent granules were shown. Immunogold staining for bacterial surface antigens (*arrows*) on these atypical forms was negative (**B**) or very weak (**C**, **D**) compared with the surface antigen expression on both typical (Fig. 1) and atypical (Fig. 2) forms of *B. pseudomallei* grown in culture media

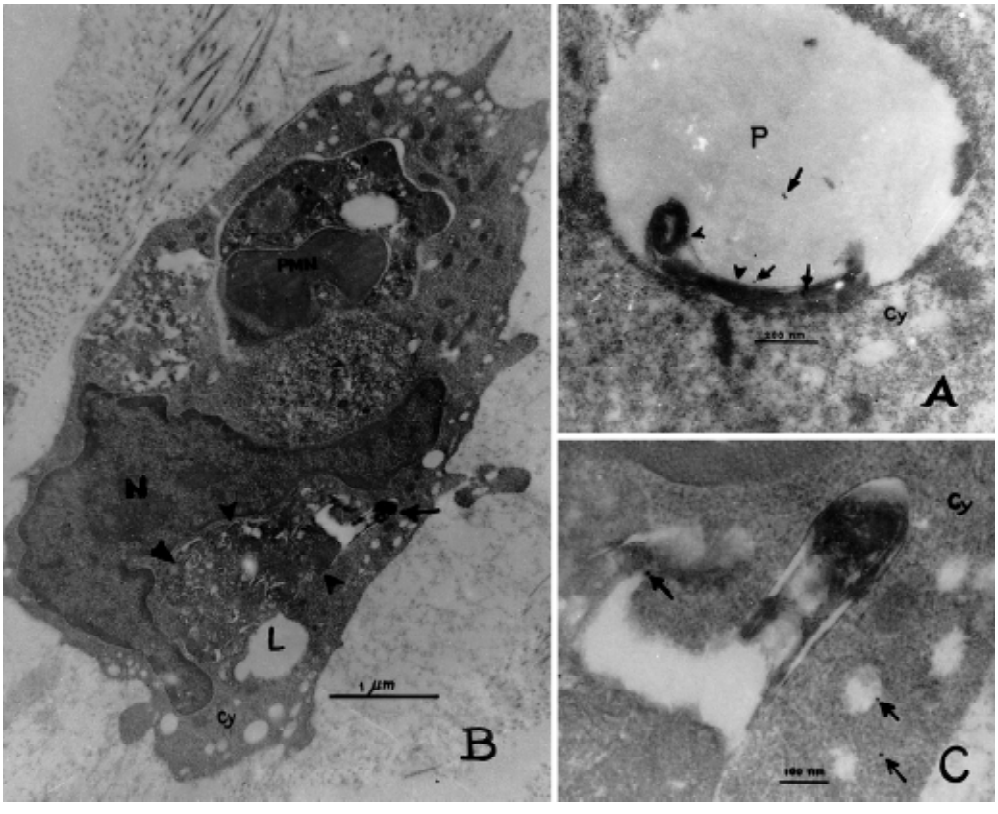

Fig. 4. Intracellular bacteria-like structures in naturally infected synovial membrane. **A** Intraphagosomal bacteria-like structures, i.e., bacteria-like structures with a portion of bacterial membrane (*arrow heads*) found in phagosomes of synovial mononuclear cells (patient 3). Weak immunogold staining for bacterial surface antigen was noted (*arrows*). **B** Intracytoplasmic bacteria-like structures, i.e., synovial mononuclear cells with interalized PMN. Large cytophagosomes (*arrow heads*) containing intracytoplasmic bacteria-like structures (*arrows*) were noted. **C** At a higher magnification, *B. pseudomallei* surface antigens were shown by a few dots of immunogold staining (*arrows*) in the areas adjacent to intracytoplasmic bacteria-like structures. *N*, nucleus; *Cy*, cytoplasm; *L*, lysosome; *P*, phagosome

(patient 2, 4). Complete bacteria-like particles were found in all four synovial specimens, both extracellularly (Fig. 3) and intracellularly (Fig. 4), including the synovial specimens taken from antibiotic-treated patients. Intracellularly, the bacteria could be in either the phagosomes (Fig. 4A) or cytoplasm (Fig. 4B) of mononuclear cells. Microcolonies of bacteria accumulated in the mononuclear cell phagosomes were also noted in three patients (Fig. 5). The bacteria found in the synovial membrane were mostly atypical, with transformed bacterial cell walls. They contained fewer

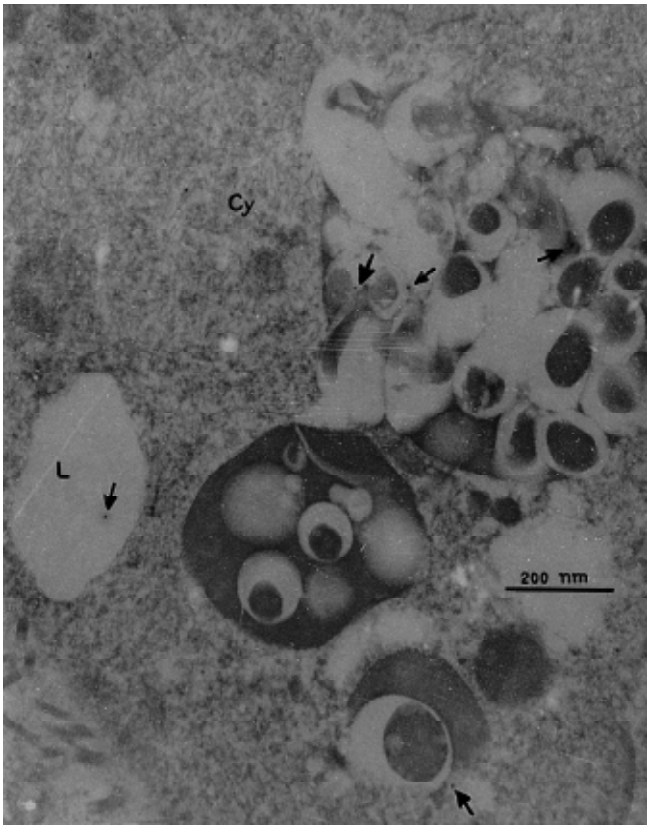

Fig. 5. A bacterial microcolony-like structure within a mononuclear cell phagosome was shown (patient 3). Bacterial structures identified in microcolonies were rather atypical, had a bull's eye appearance, but were compatible with the atypical form of *B. pseudomallei* grown in culture media shown in Fig. 2F. A few dots of immunogold staining to *B. pseudomallei* surface antigens (*arrows*) were also seen on these structures. *Cy*, cytoplasm; *L*, lysosome

electron lucent granules in the cytoplasm, which has previously been described as an ultrastructural characteristic of *B. pseudomallei*.[8] The surface antigens of *B. pseudomallei* found in synovial tissues, as shown by IGS, were weak or even absent compared with the surface antigens of *B. pseudomallei* in the positive controls.

Discussion

Our IEM study has demonstrated atypical forms of *Burkholderia pseudomallei* with suppression of bacterial surface antigens in naturally infected human synovial tissue. Abnormal *B. pseudomallei* morphology found in synovial membrane samples was not an effect of the IEM process, because the characteristic bacterial morphology was preserved in the positive controls. This change may due to direct exposure of the bacteria to the antibiotics being given (patients 1, 2, and 4). Transformation of *B. pseudomallei* membranes after exposure to kanamycin has been demonstrated in one recent experiment.[21] However, the transformation of *B. pseudomallei* was also found in patient 3, who did not receive antibiotics before synovial biopsy was performed. Thus, there are two more possibilities which might explain this phenomenon. First, it may be the effect of direct exposure of *B. pseudomallei* to certain host inflammatory cytokines responding to infection in a similar way to the pathogenic actions of host cytokines on *C. trachomatis* infection studied by Beatty et al.[20] Second, this atypical form may be a biological adaptation of *B. pseudomallei* per se, either to elude the host immune response in the patients studied, or for their survival within a limited or inappropriate environment, as shown, although very rarely, in the positive controls.

We found that the surface antigen expression of *B. pseudomallei* in the infected synovial membranes was significantly suppressed compared with the positive controls. A reduction of surface antigens was also demonstrated on a bacterium which was less transformed (Fig. 3A). Less staining of immunogold particles on the bacterial surface should not be the result of an antigenicity difference between *B. pseudomallei* in the tissues studied and those in the positive controls, because the rabbit antiserum to whole cell *B. pseudomallei* used in our IEM procedure was found to react against all strains of pathogenic *B. pseudomallei*. The mechanism that contributed to this change was not determined in our study, but it might relate, either more or less, to the transformation of their morphology. The changing metabolic function of *B. pseudomallei* during infection may be the possible mechanism.

Our IEM findings confirmed the intracellular location of *B. pseudomallei.* The organism was found not only in cellular phagosomes, but also in the cytoplasm of mononuclear phagocytes. The possibility of an intracytoplasmic location of *B. pseudomallei* was doubted in the previous study of Jones et al.[12] They observed that there was a process of phagosomal membrane degeneration in the *B. pseudomallei*-containing vacuole, but no organism could be demonstrated in the cytosol.[12] The microcolony-like structure in synovial mononuclear phagosomes found in our patients (patients 1, 2, and 3) supported the ability of *B. pseudomallei* to undergo multiplication within human phagocytic cells. Intracellular multiplication of these bacteria was reported in both the pervious experimental study[12] and EM work.[22] In the latter report, the EM study was performed on a specimen of infected human lung tissue taken during autopsy of a patient who died of septicemic melioidosis.[22] The intracytoplasmic location might provide a good niche for bacteria to stay safely away from the specific cellular killing processes of mononuclear phagocytes. In this instance, to eradicate this infection effectively, antibiotics with higher cellular penetration properties, and higher concentrations in both cellular phagosomes and cytosol should be considered.

The inflammatory cell response to *B. pseudomallei* infection found in all of our patients was predominantly in mononuclear cells. Notably, complete bacterial-like structures could not be found in polymorphonuclear neutrophils (PMNs). This supports findings from previous studies in animal models[7,11,12] which demonstrated that most organisms were killed by PMN within the first hour[12] despite

their ability to inhibit the specific intracellular killing process of phagocytes.[7,11,12] Our IEM study was performed on the infected synovial tissue taken from the secondary sites of infection. The absence of bacteria in PMN may reflect the high efficacy of the intracellular killing process of this specific phagocyte. We observed the ultrastructural characteristics of an effective intracellular killing process in some synovial mononuclear cells, but very infrequently. The EM finding of effective intracellular killing had been described in our previous IEM study on *Salmonella* septic arthritis[23] as an onion skin or finger print appearance in mononuclear phagosomes. This suggested that the bacteria could survive only in mononuclear phagocytes with defective intracellular killing ability. That the fate of *B. pseudomallei* is determined by their capsules has been postulated in a Russian experimental study on infected guinea pig lung.[24] Only *B. pseudomallei* that can escape being killed by PMN would then be able to interact with less bactericidal mononuclear phagocytes.[12] Early capsule forming bacteria can avoid phagocytosis. Bacteria that failed to form capsules in the early phase would be internalized in phagosomes. The way to survive intracellularly is either by early escape into the cytosol, with subsequent capsule formation, or by forming a capsule in the phagosome. The capsule-free *B. pseudomallei* will be killed in the phagosome by the intracellular killing process. In our IEM study, we could not show the bacterial capsule in the infected synovial membrane specimens. The bacterial capsule was seen only in bacterial colonies in the positive controls. Therefore, the fate of *B. pseudomallei* in naturally infected human tissue may depend, not only on its capsule, but also on the alteration of its biological properties.

Perivascular infiltration with inflammatory cells and endothelial cells containing bacterial products, as found in our IEM study (patients 2, and 4), suggests a role for capillary endothelial cells in *B. pseudomallei* septic arthritis. In an animal model study reported in the Russian literature,[25] they documented the tissue tropism of *B. pseudomallei* to both specific phagocytes (mononuclear phagocytes and PMN) and non-specific phagocytes (capillary endothelium, splenic lymph nodes, and reticulocytes).[25] These IEM findings, along with the clinical data from our patients, suggested that *B. pseudomallei* successfully escaped from primary sites of infection, traveled via the blood stream, and then lodged in the synovial tissue by emigration through capillary endothelial cells. Whether the organism was transported intracellularly or freely in the blood stream was not determined in our study.

In summary, our IEM study demonstrated an atypical form of *B. pseudomallei* and suppression of its surface antigens in naturally infected human synovial tissue. The intracytoplasmic location of *B. pseudomallei*, apart from the known presence in phagosomes, was also demonstrated. This biologic adaptation and its good intracellular niche may be part of the mechanism by which *B. pseudomallei* escapes from host-immune surveillance and treatment antibiotics. Future studies should attempt to define whether this biological change relates to a changing metabolic rate of *B. pseudomallei* for survival in host tissue. Study designs that can identify the factors influencing *B. pseudomallei* transformation and surface antigen synthesis may contribute to improved treatment of melioidosis.

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