MULTIFACTORIAL PATHOGENIC MECHANISMS OF *BURKHOLDERIA PSEUDOMALLEI* AS SUGGESTED FROM COMPARISON WITH *BURKHOLDERIA CEPACIA*

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Abstract. With the purpose to elucidate the pathogenesis of disease due to *Burkholderia pseudomallei* some biological and biochemical properties of this species were studied in comparison with *B. cepacia*, since the difference in the level of virulence between the two species is remarkable despite of their toxonomic closeness. *B. pseudomallei* was distinct from *B. cepacia* in the capability to grow under anaerobic conditions, with positive nitrate respiration, excretion of high-molecular polysaccharides into liquid culture, and cytotoxicity against cultured tissue cells. From these observations together with our previous finding that *B. pseudomallei* can grow and survive in an acidic environment, we suggest multifactorial mechanisms for the pathogenesis of melioidosis due to *B. pseudomallei.*

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis. Though it is an opportunistic pathogen, the infection can develop into septicemic disease with high mortality. The disease has a wide spectrum of clinical course and manifestations including latent or persistent infection with frequent reactivation. Therefore, such a spectrum covers a range of symptoms (Kanai and Kondo, 1994).

Despite this unique clinical picture, *B. pseudomallei* are essentially microorganisms living in their natural environment. Soil and surface water are their usual habitat. Transmission of infection from man to man does not occur.

This information stimulated our interest in the pathogenic factors of *B. pseudomallei*. Especially, we are concerned with a question how this species of microorganism is equipped to cause infection not always leading to disease. Disease develops only when circumstances dictate an unfavorable outcome of infection (Finlay and Falkow, 1989). In other words, our concern is how *B. pseudomallei* organisms can establish successful persistence within the host.

As an approach to this study we attempted to examine some biological and biochemical characteristics comparing between *B. pseudomallei* and *B. cepacia*. This is because that these two species are considerably different in the level of virulence despite of the close taxonomic and biochemical relatedness, the latter species being much lower.

Our basic idea is that microbial pathogenesis is usually complex and multifactorial. In this paper we comment on some factors which may be responsible for the survival and persistence of *B. pseudomallei* in the host.

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MATERIALS AND METHODS

Microorganisms

Seven local strains (UB12, UB16, UB26, UB42, UB46, UB122, UB165) of *B. pseudomallei* and five local strains (UB3, UB77, UB140, UB145, UB146) of *B. cepacia* were employed in the present study. These strains had been isolated from melioidosis cases and septicemic patients admitted in Sappasittiprasong Ubon Hospital and kept in our stock collection (Kondo *et al*, 1991). *B. pseudomallei* strains were identified on the basis of growth on the selective medium: peculiar order, characteristic colony morphology, and various biochemical tests including oxidase, fatty acid pattern determined by gas-liquid chromatography (GLC), and pH activity pattern of acid phosphatase (Kondo *et al*, 1991). Further confirmation was obtained by immunofluorescence assay using antibodies against the pro-

tein fraction and endotoxin of *B. pseudomallei* (Naigowit *et al*, 1993).

Culture medium and cultivation of micro-organisms

Five percent sheep blood agar and tryptone glucose extract (TGE) agar were employed in plastic petri dishes of 9 cm diameter. As liquid miedium, peptone yeast glucose and tryptic soy broth were used in the amount of 5 ml dispensed into a test tube. To obtain the culture filtrate, Mueller-Hinton liquid medium was employed, 1.5L being dispensed into each flask.

Anaerobic cultivation was made in the gas environment consisting of N_2, CO_2 and H_2 in the proportion of 85%, 5% and 10%, respectively.

Preparation of cell-free extract of *B. pseudomallei*

The cells of *B. pseudomallei* harvested from the overnight culture TGE agar were suspended in 100 ml of 0.01 M EDTA solution in 0.1M Tris buffer pH 7.2 and subjected to 15 cycles of freezing and thawing in the temperature range between -20°C and 35°C, followed by sonicated for 3 minutes to promote disruption and dispersion.

The extract obtained was centrifuged at 2,000 rpm for 10 minutes. The resulting supernatant was subjected to recentrifugation at 5,000 rpm for 20 minutes. The supernatant was preserved and the precipitate was washed with 30 ml of Tris buffer by centrifugation at 5,000 rpm for 20 minutes. The washings were combined with the preserved supernatant. The pooled supernatant was centrifuged at 12,000 rpm for 30 minutes. The resulting precipitate was resuspended in 20 ml of water and washed by centrifugation under the same conditions as before. The supernatant was subjected to ultracentrifugation at 100,000*g* for 60 minutes to yield precipitate and supernatant. The precipitate fraction was designated as Fraction D and the supernatant fraction was designated as Fraction E, which was then separated into 3 subfractions of F, G and H by DEAE-column chromatography as described later.

Fractionation of culture filtrate and cell-free extract

After termination of incubation each culture flask was added with formalin to a concentration of 0.35% and left to stand overnight at 37°C. The sterility test showed no detectable living bacilli at this time. The bacterial cells were removed therefrom by centrifugation at 10,000 rpm for 30 minutes. The supernatant was concentrated to a 1 : 10 volume by a rotary evaporator. The concentrate was added with an equal amount of a saturated amonium sulfate solution and left to stand overnight at 4°C. The resulting precipitate was collected by centrifugation at 4,000 rpm for 30 minutes. The precipitate was dissolved in 150 ml of 0.067M phosphate buffer, pH 7, and the half saturation procedure with ammonium sulfate was conducted twice more. The final precipitate was dissolved in 100 ml of distilled water and dialyzed against water at 4°C for 4 to 7 days. Then the dialysis bag was placed in Ficol powder (Sigma Chemical Co) to concentrate the inside solution to about 60 ml.

The above ammonium sulfate precipiate fraction was then applied to a Sephadex G-75 column (2 × 30 cm) equilibrated and eluted with water. The loaded amount was 20 ml, and the eluates were collected in 3 g amounts. A 20 µl portion from each tube was employed for determination of protein and sugar. Pooled eluate fractions of each peak were lyophilized.

The Sephadex fractions were then applied to a DEAE-collulose column (preswallen microgranular Anion Exchanger DE 52, Whatman Bio Systems Ltd, England) of 1.5 × 11 cm equilibrated with 0.025 M Tris-HCl buffer pH 7.1. The loaded amount of the Sephadex fractions was 100 mg dry weight each. Elution was made stepwise first with 100 ml of 0.025 M Tris-HCl buffer, pH 7.1, then 0.1 M NaCl, and finally with 0.5 M NaCl in 0.025 M Tris-HCl buffer. The fraction size was 4g each. The eluates in each peak depicted by protein and sugar determinations were pooled, dialyzed, and lyophilized. These pooled eluate fractions were designated as A, B and C.

The fractionation of cell-free extract was conducted in almost the same procedure as above. The final supernatant (Fraction E as described above) was dialyzed against water at 4°C for 3 days, and then concentrated to a volume of 28 ml with Ficol. A 3 ml portion of this material was set by and lyophilized. The remaining portion was divided into equal halves, and each portion was fractionated by column chromatography with DEAE-sepharose (1.5 × 10 cm). Before use, DEAE-sepharose (Pharmacia, Sweden) was equilibrated with 0.025 M

Tris-HCl buffer pH 7.4. Elution was made stepwise first with the above buffer, then 0.1 M NaCl, and finally with 0.5 M NaCl, each time using 90 ml. The eluate size was 4 ml and was assayed for protein and sugar levels. The pooled eluate fraction of the peaks were designated F, G and H.

Determination of protein and sugar content

Protein levels were determined by the Lowry method or with Bio Rad protein assay reagent (Bio Rad Chemical Division, Richmond, CA). Bovine serum albumin (Fraction V, Sigma) was used as a protein standard. Sugar determination was made by the phenol-H_2O method as follows: A sample of 20 µl was placed in a test tube of 1.2 × 7 cm (Corning) with 780 µl of water, and added with 20 µl of 80% phenol. This mixture was then treated with 2 ml of concentrated sulfuric acid. After the temperature went down to 25-30°C, the developed color was read with a Coleman spectrophotometer set at 490 nm. Glucose was used as a sugar standard.

Nitrate reductase test

One loop of overnight culture colony was suspended in 3 drops of water and added with 2 ml of 0.01 M $NaNO_3$ solution. After standing at 37°C for 2 hours with occasional agitation, one drop of a 1 : 2 dilution of HCl, one drop of 0.2 % sulfanilamide and one drop of 0.1 % N-naphthylethylene diamine dihydrochloride was added to the incubation mixture. When red or pink color developed, the reaction was read as positive. The mixture of no color change was valid of negative reaction after comfirming the development of red color by adding of zinc powder.

Cytotoxicity test

Cytotoxicity assay was performed on culture filtrates and cell-free extracts using HEp-2 cells in a microtiter system. To 0.05 ml of cell culture, 0.05 ml of 2-fold serial dilutions of bacterial filtrates or extracts were added in duplicated and inoculated for 24-48 hours. For negative control, 0.05 ml of phosphate-buffered saline (PBS, pH 7.2) or tryptic soy broth were added. A cytopathic change of HEp-2 cells was observed before and after stained with 0.13% crystal violet in 5% ethanol-2% formalin-PBS.

Electronmicroscopy

Mice weighing around 35 g were injected intraperitoneally with 2 ml of 5% casein solution. The next morning, they were further injected intraperitoneally with 2 ml of the suspension of *B. pseudomallei* (UB26) or of *B. cepacia* (UB77). The density (OD) of the former was 0.52 × 20 and the latter 0.50 × 20 at 420 nm. Two hours after the injection, peritoneal fluid was washed out with PBS. Then, the pooled fluid was centrifuged at 1,000 RPM for 5 minutes to collect exudate cells. The collected cells were washed 2 times with PBS.

The harvested cells were added with the 1 : 1 mixture of 2.5% para-formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After being left to stand for 2 hours, the cells were washed 3 times with PBS by centrifugation.

Thus treated cell samples were postfixed in cold 1% OsO_4 in the same buffer and then stained with 0.5% uranyl acetate in acetate-veronal buffer, pH 5.0. They were then dehydrated in graded ethanol and embedded in Epon 812. Ultrathin sections were cut on an LKB ultratome and stained with uranyl acetate followed by lead citrate. The sections were observed with Hitachi H-600 electron microscope at 75 kv.

RESULTS

Anaerobic growth of *B. pseudomallei* and nitrate reductase

Strain UB16 and UB26 of *B. pseudomallei,* which had been maintained on TGE agar, were inoculated in blood agar plates and two kinds of broth as described above. The inoculated media were then placed in the gas environment consisting of N_2, CO_2, H_2 in the proportion of 85%, 5%, and 10%, respectively. Daily observation was made to follow the growth of the inoculum for 19 days. The next day of incubation, a few colonies appeared on blood agar plates inoculated with UB26. Seven days later, enough growth developed on the plates of both UB16 and UB26. Likewise, the inoculated broth with each of the strains exhibited good turbidity in eight days incubation. When turbid culture was subcultured on blood agar plates under aerobic conditions, abundant growth developed in-

dicating good viability of anaerobic broth culture. The growth on blood agar plates of anaerobic culture was also subcultured successfully on the agar plates under aerobic conditions.

The same design of experiment was conducted with strain UB77 and UB145 of *B. cepacia*. In this case, however, no turbidity developed in the inoculated broth under anaerobic conditions, and no colonies appeared when subcultured onto blood agar plates under aerobic conditions. This observation indicates that *B. cepacia* cannot grow and survive in the broth under anaerobic conditions. As for the case with blood agar plates under anaerobic conditions, only UB77 showed a limitted growth, but the subculture to the same medium under aerobic condition produced no colonies thus indicating the unsuccessful survival in the anaerobic growth.

Along with the above experiments, nitrate reductase activity was compared between *B. pseudomallei* and *B. cepacia*, each employing five strains. The result was so clear-cut all of the *pseudomallei* strains were positive and all of the *cepacia* strain were negative. The results are sumarized in Table 1.

Excretion of high-molecular polysaccharides into the growth environment by *B. pseudomallei*

During our study searching for biologically active substances in the culture filtrate of *B. pseudomallei* comparing with *B. cepacia*, we have been encountering with the different fractionation patterns of protein, sugar and lipid analysis between the two species. One of such patterns was concerned with the polysaccharides of high molecular weight excreted into the environment. Fig 1 shows one example of the difference. In this figure, the gel-filtration patterns on a sephadex G-75 column (1.5 × 10 cm) loaded with the precipitate fracion by half-saturation with ammonium sulfate from the culture filtrate (Meuller Hinton medium) are compared between *B. pseudomallei* (UB12) and *B. cepacia* (UB77). Elution was made as described before in the same conditions to both strains. The elution pattern was depicted by the determination of protein and sugar amount (Fig 1).

In the case of *B. pseudomallei*, the pattern was roughly divided into 3 eluate fractions, I, II, and III. Fraction I as eluted in the void volume is higher than 50,000 in the molecular weight. However, in the pattern of *B. cepacia*, the corresponding fraction to I of *B. pseudomallei* did not appear, but only the fractions corresponding to II and III of lower molecular weight are shown.

Column chromatography by DEAE-cellulose with dried 100 mg sample could separate almost completely the sugar portion from Fraction I of *B. pseudomallei* (Fig 2) and Fraction II of *B. cepacia* (data not shown) to the void volume. DEAE col-

Table 1

Anaerobic growth of *B. pseudomallei* as facultative anaerobes.

Species strains	Growth				Nitrate reductase
	Anaerobic growth at $85\%N_2 + 5\%CO_2 + 10\%H_2$		Aerobic subculture on agar*		
	Agar* (A)	Broth** (B)	From A	From B	
B. pseudomallei					
UB16	+	+	+	+	+
UB26	+	+	+	+	+
B. cepacia					
UB77	+	-	-	-	-
UB145	-	-	-	-	-

* 5% sheep blood agar
** Peptone yeast glucose and tryptic soy broth
\+ Occurrence of growth or of enzymatic reaction

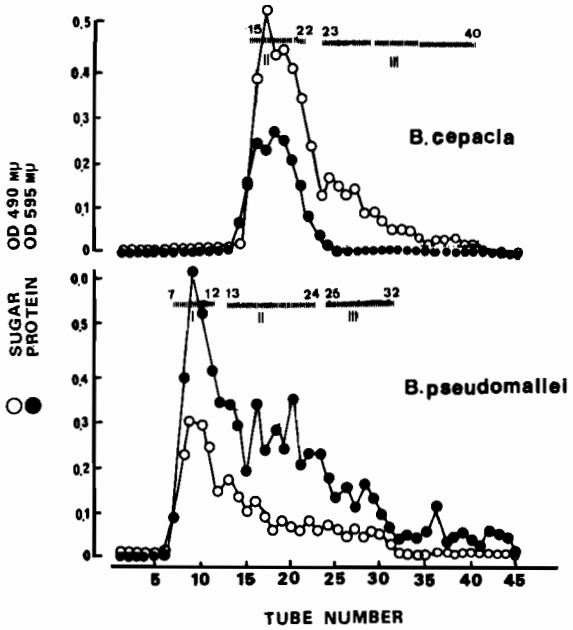

Fig 1–Gel-filtration patterns on a sephadex G-75 column (1.5 × 10 cm) loaded with the precipate fractions by half-saturation with ammonium sulfate of the culture filtrates of *B. pseudomallei* (UB12) and *B. cepacia* (UB77).

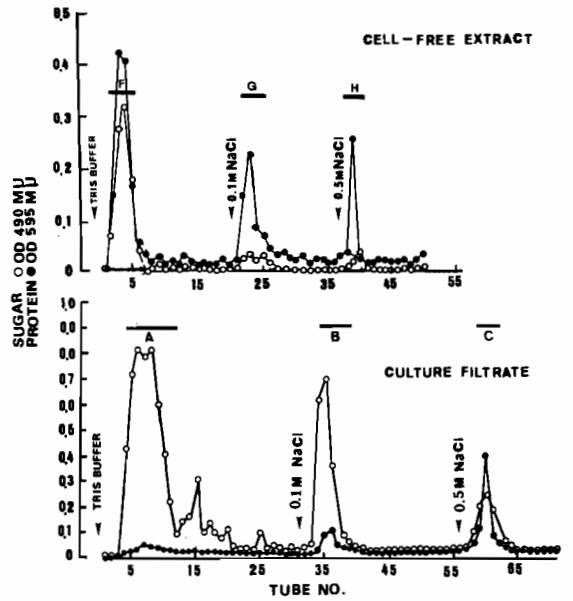

Fig 2–DEAE-eluted fractions of *B. pseudomallei* which were subjected to cytotoxicity test as shown in Table 2.

umn fractions from *B. pseudomallei* were designated UB12-I-A, -B and -C. The yield was 29.6 mg, 16.7 mg and 11.4 mg in lyophilized weight, respectively. The fractions from *B. capacia* were designated UB77-II-A and -B. Their weights were 77.5 mg and 1.5 mg. Thus purified sugar components were analysed to show that sugar content of the *B. pseudomallei* (UB12-I -A) was 77.5 μg per 100 μg and protein content was 5 μg per 100 μg, and that sugar content of the *B. cepacia* (UB77-II-A) was 75 μg per 100 μg and protein content was 3 μg per 100 μg.

This sort of results were confirmed in the gel-filtration experiments with the other UB16 strains of *B. pseudomallei*.

Cytotoxicity of the *B. pseudomallei* components separated from the culture filtrates and cell-free extract

The results with 50 μg (lyophilized weight) samples per well are shown in Table 2. The control experiments by addition of PBS or tryptic soy broth to HEp-2 cell culture did not bring about any cytopathic effect. Meanwhile, 80 to 100 % of HEp-2 cells exposed to each DEAE fractions of culture filtrates and cell-tree extracts of *B. pseudomallei* showed cytopathic morphological changes from spindle form to round form. Such cell rounding occured only in 30% of cell cultures exposed to the corresponding fractions of *B. cepacia*.

Electronmicroscopy

This ultrastructural study demonstrated clearly that both *Burkholderia* species were intracellular microorganisms making mononuclear phagocytes as host cells (Fig 3). One host cell can have many *Burkholderia* organisms within the phagosomes (Fig 3A, Fig 3D).

When the host mononuclear cell came into degeneration as indicated by vacuolated cytoplasm, *Burkholderia pseudomallei* escaped from the damaged phagosome to be scattered into the cytoplasm (Fig 3B).

Within the bacterial bodies of both species, electron-dense and electron-transparent round bodies are often observed. In some longitudinally-sectioned cells, the fibrous chromosomal structure

Table 2

Cytotoxicity of Sephadex-DEAE fractions separated from the culture supernatant and cell-free extract of *B. pseudomallei* and *B. cepacia* (exposure to 50 μg per well in cell culture).

Species	Microbial fractions	Cytotoxicity % destruction of cultured cells
B. pseudomallei UB12	Culture filtrate	
	DEAE fraction -A	80
	-B	80
	-C	100
	Cell extract	
	100,000g precipitate -D	100
	DEAE fraction of	
	100,000g supernatant -F	100
	-G	90
	-H	50
B. cepacia UB77	Culture filtrate (Sephadex fraction)	30
	Culture filtrate	
	DEAE fraction -A	30
Control	Cell culture only	0

is seen. However, the morphological differentiation between *B. pseudomallei* and *B. cepacia* is difficult from the present study.

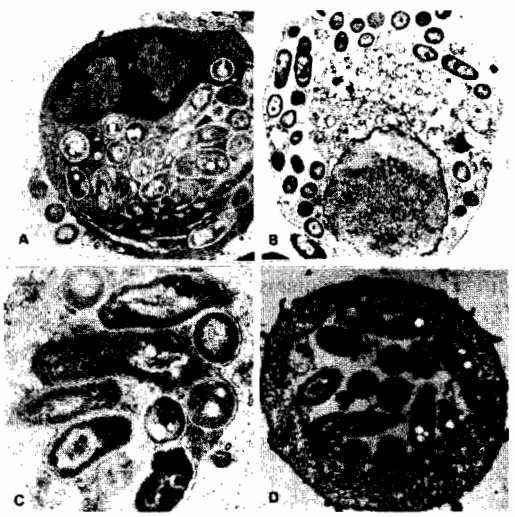

Fig 3–Intracellular *B. pseudomallei* (A,B,C) and *B. cepacia* (D) within mouse peritoneal exudate cells (monocytes).

DISCUSSION

Pseudomonas species have been generally described as aerobic microorganisms (Gilardi, 1986). However, Zannoni (1989) pointed out that pathogenic *Pseudomonas* were equipped with the metabolic machinery of anaerobic respiration for energy generation. For example, *P. aeruginosa* shows L-arginine-dependent anaerobic growth (Wauven *et al*, 1984). *P. pseudomallei* (*B. pseudomallei*) has been known to have nitrate reductase activity for anaerobic respiration, which can differentiate this species from *B. cepacia* (Ashdown, 1992).

Our study reported here provided definite evidence for the above by the culture experiment under anaerobic conditions and concomitant observation of nitrate reduction activity. Thus, *B. pseudomallei* is a facultative anaerobic microorganism. They can not only multiply in an oxygen-rich environment, but also they can grow and survive under oxygen-limited conditions by change-over of their metabolic pattern from the aerobic to anaerobic mechanism.

The capability of *B. pseudomallei* for this adap-

tation to gas conditions may favor their survival in the natural environment and also in the lesions of infected host tissue. *B. pseudomallei* is an inhabitant of the soil and surface water. Infection with this species occurs most frequently among farmers working in rice paddies in the rainy season.

In rice paddies, the *B. pseudomallei* organisms have a good chance of multiplication not only in the aerobic layer of around 20 mm thickness prevailing under the surface water but also in the anaerobic layer located below the aerobic layer. Ammonium sulfate as fertilizer will be converted into HNO_3 by the action of nitrifying bacteria in the aerobic layer and the resulting HNO_3 will move to the anaerobic layer to serve the nitrate respiration of *B. pseudomallei* cells. In the dry season, they are considered to survive in the deep soil of poor aeration until the return of the rainy season.

Within the infected host, *B. pseudomallei* produces abscesses and granulomatous lesions of the tuberculous type where a gas environment of lower oxygen tension is prevailing. It is reasonable to consider that *B. pseudomallei* cells can survive in such an environment by taking advantage of their anaerobic metabolism. Actually, latent infection, chronic melioidosis, and recrudescent melioidosis have been reported as more common types of disease than septicemic cases (Kingston, 1991; Carruthers, 1981).

Judging from the above, the anaerobic pattern of metabolism in *B. pseudomallei* can not be overemphasized in the pathogenesis of melioidosis.

The presence of a slime layer around the cells of *B. pseudomallei* has been noted from the gross appearance on laboratory media (Jayanetra, 1989) and also from the cytochemistry with ruthenium red as a polysaccharide reagent (Kaliev *et al*, 1990; Vorachit *et al*, 1993).

In this context, our present observation that *B. pseudomallei* excreted high-molecular weight polysaccharides into the culture environment but *B. cepacia* did not, may be suggestive of a basis for the difference of virulence between these two species. It is difficult, however, to decide whether *B. cepacia* cannot synthesize such high molecular weight polysaccarides or lacks an excretion mechanism for the synthesized molecules. The latter case would not be excluded, since we have a previous observation that endotoxins were isolated from the harvested cells of *B. cepacia* but not from the culture filtrate unlike the case of *B. pseudomallei* (Iwasa *et al*, 1992).

In the cytotoxicity test reported in this paper the fractions obtained from the culture filtrate or cell-free extract were employed on the same weight basis (dried 50 μg) so that the quantitative comparison could be possible between *B. pseudomallei* and *B. cepacia*. The cytotoxicity was evaluated by the morphological change of cultured cells from spindle form to round form identified by cystal violet staining. Percent destruction of the fraction-exposed cells was counted and shown in Table 2. From this table it will be seen that the fractions from *B. pseudomallei* were more cytotoxic than those of *B. cepacia*. When the amount of *B. cepacia* fractions employed was 100 μg per well, the percent destruction value increase from 30 to 60 or 90.

All the fractions of *B. pseudomallei* brought about the complete (100%) destruction of the cultured-cell population.

The mechanism of the cytotoxicity as observed here is not known. Ashdown and Hochler (1990) searched for extracellular, biologically active substances elaborated by clinical isolates of *B. pseudomallei* as candidates for virulence factors. Most of the test strains were positive for lecithinase, lipase and protease but none was positive for elastase. We also observed the presence of phospholipase A and C as a membrane-active agent, but there was no difference in the level of enzymatic activity between *B. pseudomallei* and *B. cepacia*.

As for lipopolysaccharides (LPS : endotoxins), Iwasa *et al* (1992) showed that LPS of *B. cepacia* is less toxic than that of *B. pseudomallei* when tested by mouse-body weight decreasing test. However, there is no evidence to show that LPS is responsible for the cytotoxicity in the present systems.

Our electronmicroscopy demonstrated clearly that both *B. pseudomallei* and *B. cepacia* are intracellular parasites located within the phagosomes of mononuclear cells.

Since the observation was made only at the early stage of infection without time-course follow-up, we can say nothing about the different cyto-toxicity between the two species from the present morphological study.

In connection with this matter, however, we would like to emphasize our previous observation the *B. pseudomallei* can initiate the growth at acidic

environment such as pH 4.5, but *B. cepacia* can not (Dejsirilert *et al*, 1991). It has long been known that pH within phagosomes is acidic, 4.7 to 5.5 (Sprich, 1956).

In any case, microbial pathogenesis is usually complex and multifactorial, and can not be explained by a single factor. The observations reported in this paper are concerned with putative pathogenic factors to explain the growth or survival potential of *B. pseudomallei* within the host, and to explain the difference of virulence from *B. cepacia*.

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