Survival of *Pseudomonas pseudomallei* in human phagocytes

SURAPEE PRUKSACHARTVUTHI, NALINEE ASWAPOKEE and KLEOPHANT THANKERNGPOL

Departments of Medicine and Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

Summary. *Pseudomonas pseudomallei* causes the disease melioidosis, with protean manifestations, protracted clinical course and unpredictable response to antimicrobial treatment. Intracellular location of the organism is suspected to be the cause of these properties. This study was undertaken to examine the intracellular growth of this bacterium. Intracellular growth and survival was assessed at different time intervals, by Gram's stain and electronmicroscopic examination. During the first 5 h, the numbers of *P. pseudomallei* within phagocytes did not change significantly. By 18–21 h, gram-stained preparations revealed that *P. pseudomallei* cells completely filled the phagocytes and electronmicroscopy showed evidence of binary fission. During that time the number of cfu of *P. pseudomallei* growing simultaneously in vitro increased by log10 2–3. The phagocytes remained viable throughout the observation period and retained their capacity to produce an oxidative burst for the first hour of incubation. The ability of *P. pseudomallei* to survive and multiply in phagocytes shows that it is a facultative intracellular bacterium. This finding is relevant to the selection of antimicrobial regimens, and the management of the disease.

Introduction

*Pseudomonas pseudomallei* is a small, motile, gram-negative, non-acid-fast, non-spore-bearing bacillus that causes the serious and frequently fatal disease, melioidosis (Groves, 1979; Thin, 1981). Although much has been learned about the epidemiology, clinical manifestations and course of the disease, and its response to antimicrobial agents (Proceedings, 1985), the difficulty of eradicating the infection, its protracted course, and certain features of the disease point towards facultative intracellular growth of the organism. Moreover, it is postulated that after the initial phase of infection, *P. pseudomallei* can persist in a dormant stage in macrophages for months or years. Recurrences of the disease have been encountered frequently, even after many years (Groves, 1979; Patamasucon *et al.*, 1982). The longest latent period, 24 years, was reported in a 56-year-old man (Kingston, 1971). Previous study has shown that the organism can reside in the mononuclear cells of man (Sanford, 1979).

Because of the implications of its capacity for intracellular growth and survival for the management of the disease, the interaction of *P. pseudomallei* with human phagocytes was studied further.

Materials and methods

Preparation of bacteria

Three recent clinical isolates of *P. pseudomallei*, nos. 0035, 0037, 0038, cultured respectively from pus, sputum and blood of patients in hospital, were used. These strains were kept at −70°C in Brain Heart Infusion Broth, pH 7-4 (Difco Inc, Detroit, USA), with glycerol 10%. Before the experiment, the organisms were subcultured on blood agar plates incubated at 35°C for 18–24 h and then grown in Trypticase Soy Broth, pH 7.3 (Difco). They were then washed twice with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Inc, Grand Island, NY, USA) and adjusted to $5 \times 10^8$ cfu/ml in RPMI-1640 medium.

Preparation of polymorphonuclear and mononuclear leucocytes

Polymorphonuclear (PMNL) and mononuclear (MNL) leucocytes were separated from fresh whole blood by a method modified from that of Böyum (1968). Briefly, heparinised blood was obtained from healthy donors who were not on any medication. Theuffy coat was collected.
and diluted 1 in 2 in phosphate-buffered saline (PBS), pH 7.2. Then 16 ml of the cell suspension was layered smoothly on to 3 ml of Ficoll-Hipaque (Pharmacia) and centrifuged at 1000 g for 20 min at 25°C. Mononuclear cells from the interface were collected, and washed three times with cold RPMI-1640 medium by centrifugation at 800 g for 5 min at 4°C. Residual erythrocytes in the pellet were lysed with ice-cold NH₄Cl (0.87% w/v in sterile distilled water) for 10 min at 4°C. After centrifugation, PMNLs from the deposit were washed three times in cold RPMI-1640 medium. The cells were confirmed as more than 95% viable by the trypan blue exclusion test. The concentration of PMNLs or MNLs was adjusted to 10⁷ cells/ml in RPMI-1640 medium and suspensions were used immediately.

**Phagocytosis**

Phagocytic mixtures consisted of 0.5 ml of PMNL or MNL (10⁷ cells/ml), 0.1 ml of P. pseudomallei suspension (5 x 10⁸ cfu/ml), 0.1 ml of serum and RPMI-1640 medium to give a final volume of 1.0 ml. The mixtures were incubated in the 37°C water bath. After 15 min, phagocytosis was stopped by adding cold RPMI-1640 medium and centrifuging at 800 g for 5 min at 4°C. The bacteria-infected PMNL or MNL pellet was washed five times with cold RPMI-1640 medium to remove non-leucocyte-associated bacteria. The bacteria-infected PMNL or MNL were suspended in 1.0 ml of RPMI-1640 medium (Verhoef et al., 1977; Lorian and Atkinson, 1984; Rozenberg-Arska et al., 1985).

**Detection of intracellular location of bacteria**

The bacteria-infected PMNL or MNL suspensions were incubated in a water bath at 37°C for 0, 0.5, 1, 2, 3, 4, 5, 18 and 21 h. After the indicated time intervals, the following steps were undertaken:

(a) A 0.1-ml volume of sample was removed, diluted in cold sterile distilled water and mixed on a Vortex mixer to disrupt the phagocytes. Viable counts of bacterial cfu were determined by spreading 10-μl volumes from standard 10-fold serial dilutions on blood-agar plates which were then incubated at 37°C for 24 h (Lorian and Atkinson, 1984; Rozenberg-Arska et al., 1985).

(b) Volumes of 0.4 ml of sample were centrifuged at 800 g for 5 min at 4°C. The pellets were spread on glass slides and the smears were stained by Gram's method after heat fixation.

(c) Electronmicroscopic examination was performed by a modification of the method of Rozenberg-Arska et al. (1985). Briefly, 0.5 ml of sample was mixed with an equal volume of glutaraldehyde, 0.5% v/v in 0.1 M phosphate buffer, pH 7.4, at 0°C. The mixtures were then centrifuged at 600 g for 5 min at 4°C. The supernate was removed and the pellets were suspended in 0.5 ml of human plasma at 0°C and centrifuged at 600 g for 5 min at 4°C. The supernate was again removed, and the pellets were overlaid with glutaraldehyde, 3% v/v in 0.1 M phosphate buffer, pH 7-0, for 1 h. The pellets were then washed twice with 0.1 M PBS at 0°C, post-fixed in osmium tetroxide, 1% w/v in PBS, for 1 h and embedded in Epon resin. Ultrathin sections (60-90 μm) were double-stained with uranyl acetate and lead citrate. The sections were examined with a Jeol JEM 100 SX transmission electron-microscope at 80 KV.

**Tests for phagocyte viability and function**

**Phagocyte viability.** Phagocyte viability was determined by exclusion of trypan blue at each time interval.

**Phagocytic oxidative metabolic function.** The oxidative metabolism of phagocytes was determined by nitroblue tetrazolium (NBT) reduction by a method modified from that of Nathan et al. (1969) and Wilson et al. (1980). Briefly, 10-μl volumes of samples at 0, 0.5, 1, 2, 3, 4, 5, 18 and 21 h were mixed with an equal volume of NBT, 0.1% w/v, on a glass slide. The slide was incubated at 37°C for 30 min in a moist chamber and examined microscopically. Cells which were stained a deep blue colour were read as a positive NBT test. Cells with diffuse fine granular deposits were read as negative.

**The in-vitro viability of P. pseudomallei**

The viability of P. pseudomallei was determined as a control by incubating a suspension of the bacterium at a concentration of 10⁵ cfu/ml in RPMI-1640 medium in a 37°C water bath at 0, 0.5, 1, 2, 3, 4, 5, 18 and 21 h. The counts of cfu were performed at each time interval by the standard 10-fold serial dilution method.

**Results**

To detect the intracellular location of P. pseudomallei, the number of viable bacteria within phagocytes was counted at different time intervals. By electronmicroscopy, it was found that at 0 h after mixing phagocytes and bacteria, both MNL and PMNL contained 1–2 bacteria (fig. 1). At 0.5, 1, 2, 3, 4, and 5 h, the numbers of P. pseudomallei detected in MNL and PMNL remained the same, i.e., c. 1–2 cells/phagocyte. However, by 18 and 21 h, the organisms had proliferated and filled the phagocytes. Evidence of intracellular replication was shown by the appearance of binary fission stages and some residual connection between the organism on electronmicroscopic examination (fig. 2). The simultaneous cell count by standard 10-fold serial dilution showed that the numbers of cfu of viable bacteria released from PMNL were 2.5 x 10⁵, 5.2 x 10⁴, 1.0 x 10⁵, 2.3 x 10⁵, 9.1 x 10⁴, 2.8 x 10⁵ and 4.1 x 10⁵ cfu/ml respectively and from MNL were 3.3 x 10⁵, 1.0 x 10⁵, 8.8 x 10⁴, 1.1 x 10⁵, 2.5 x 10⁵, 1.8 x 10⁵ and 3.0 x 10⁵ cfu/ml respectively at times of 0, 0.5, 1, 2, 3, 4 and 5 h. Fig. 3
Fig. 1. Electronmicroscopic appearance of human phagocytes engulfing *P. pseudomallei* (arrowed) after incubation for 1 h; uranyl acetate and lead citrate; $\times$ 15 750.

Fig. 2. Electronmicroscopic appearance of human phagocytes with intracellular dividing *P. pseudomallei* showing residual membrane contact (arrowed); uranyl acetate and lead citrate; $\times$ 15 750.
summarises the in-vitro results. At 18 and 21 h, the numbers of cfu of *P. pseudomallei* increased to $9.1 \times 10^6$ and $5.2 \times 10^7$ cfu/ml, respectively, for PMNL and to $8.3 \times 10^6$ and $1.5 \times 10^7$ cfu/ml respectively for MNL.

The viability of *P. pseudomallei* was checked by in-vitro culture in RPMI-1640 medium, and counts of the growth were performed at 0, 0.5, 1, 2, 3, 4, 5, 18 and 21 h. The results are shown in the table. From 0 to 5 h, the inoculum of $1.0 \times 10^5$ cfu/ml increased to $9.1 \times 10^7$ cfu/ml. By 18 and 21 h, the final bacterial count reached $>10^{12}$ cfu/ml.

The viability of phagocytes was demonstrated by using trypan blue as stated. More than 95% of phagocytes were viable at 0–5 h and more than 80% at 18–21 h by this technique. Tests of oxidative metabolism as detected by NBT reduction remained positive for up to 1 h and became negative in older cultures.

**Discussion**

This study showed that *P. pseudomallei* remained undigested and multiplied within human phagocytes. In the first 5 h the number of viable phagocyte-associated bacteria did not increase significantly while that of the in-vitro control bacterial culture showed a logarithmic increase. The reduction of NBT by phagocytes remained positive for the first hour of incubation but later became negative, although more than 95% of the phagocytes remained viable in the control culture. This means that the cessation of the oxidative metabolic burst was independent of phagocytic ability.

The precise mechanism by which *P. pseudomallei* survives within human phagocytes is not known. NBT reduction is closely related to metabolic events in the respiratory burst following ingestion,


**Table.** The growth of *P. pseudomallei* in RPMI-1640 medium

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Mean number (SD) of <em>P. pseudomallei</em> (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0 (1.3) x 10^6</td>
</tr>
<tr>
<td>0.5</td>
<td>3.4 (2.1) x 10^6</td>
</tr>
<tr>
<td>1</td>
<td>6.7 (1.8) x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>9.2 (3.5) x 10^6</td>
</tr>
<tr>
<td>3</td>
<td>2.5 (1.2) x 10^7</td>
</tr>
<tr>
<td>4</td>
<td>1.8 (2.6) x 10^7</td>
</tr>
<tr>
<td>5</td>
<td>9.3 (2.4) x 10^7</td>
</tr>
<tr>
<td>18</td>
<td>&gt; 10^12</td>
</tr>
<tr>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.D.

including increased H₂O₂ and superoxide radical formation; therefore, this method is useful in demonstrating the overall phagocytic activity. The phagocytes used in our system had normal oxidative function as shown by the positive NBT reduction at 0 h. This function was also intact at 0.5 and 1 h but the test was negative at 2, 3, 4 and 5 h, indicating the cessation of the metabolic process. At this time, *P. pseudomallei* cells were still viable and subsequently proliferated after the end of the metabolic burst. Thus *P. pseudomallei* was either able to resist killing by the phagocytic oxidative process or perhaps to suppress the continuous process of the oxidative metabolic burst. At the end of the exponential phase, *P. pseudomallei* in phagocytes proliferated to the same numbers as in the cell-free medium. This is evidence that *P. pseudomallei* is able to survive and multiply within human phagocytes.

There are various intracellular functions which make up the antimicrobial armamentarium, such as acid pH of phagosome, lysozyme, lactoferrin, cationic proteins, myeloperoxidase-halogenation system, H₂O₂, superoxide radical, hydroxyl radical and singlet oxygen. The purpose of this study was the preliminary demonstration of the intracellular survival and multiplication of *P. pseudomallei* and the interaction of this bacterium with specific cellular processes was not examined.

To survive intracellular destruction, many pathogenic bacteria have developed adaptive mechanisms (Densen and Mandell, 1980). Thus some bacteria provoke only a reduced oxidative burst after leucocytic ingestion. This will reduce their exposure to the potent microbicidal activity of the various reactive oxygen intermediates that are released by phagocytes during the oxidative burst (Babior, 1978). Although the exact mechanism by which *P. pseudomallei* survived the intracellular digestion is not known, it is evident that the organism survived the 1-h phagocytic oxidative burst and proliferated thereafter.

The results of this study may explain several peculiar clinical manifestations and the natural history of this disease. The ability of *P. pseudomallei* to survive and multiply in phagocytes may be the cause of the difficulty of treating melioidosis in spite of the fact that antimicrobial agents are effective against the organism *in vitro*, the protracted course of the disease and the frequent recurrences when the duration of treatment is not long enough. The antimicrobial agents should penetrate well into leucocytes so that the intracellular concentration achieved is above the minimal bactericidal level (Aswapokee et al., 1985; Aswapokee, 1986a, b, 1988).

In a previous study (Bremmelgaard et al., 1982), it was shown that 2 weeks after admission of a 64-year-old male case of melioidosis, examination of neutrophil granulocyte function showed that chemotaxis was normal, but phagocytosis was increased and bactericidal capacity slightly decreased. Three and a half weeks after admission, monocyte chemotaxis and phagocytosis were normal; the bactericidal capacity was now depressed. The mechanisms responsible for these findings are not known. It is necessary to investigate the possibility that some substances or properties of this bacterium inhibit the intracellular killing process, such as reduction of the oxidative burst (Densen and Mandell, 1980) or inhibition of phagosome-lysosome fusion as occurs with other organisms such as *Mycobacterium tuberculosis*, *Toxoplasma gondii* or *Legionella pneumophila* (Armstrong and Hart, 1971; Jones and Hirsch, 1972; Hart and Armstrong, 1974; Goren, 1977; Horwitz, 1983).

This study indicates that *P. pseudomallei* is a facultative intracellular bacterium. Thus, treatment should be tailored to the nature of the organism to achieve the most effective result. Other properties of *P. pseudomallei* such as the ability to lie dormant intracellularly, which may also influence the results of treatment, require further investigation.

We thank Ms Ratsara Preungsuwan for the preparation of the manuscript.
REFERENCES


