Experimental infection of normal and immunosuppressed pigs with *Pseudomonas pseudomallei*

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SUMMARY: A single dose of 5x10^4 bacilli of *Pseudomonas pseudomallei* by intratracheal injection resulted in acute (21 cases) or chronic (19 cases) melioidosis in 40 of 48 pigs. Fifteen (10 acute and 5 chronic) had been immunosuppressed by cyclophosphamide before inoculation. The major clinical signs were initial fever, marked neutrophilia and, in the acute cases, respiratory distress. There were no signs of the nasal and ocular discharge, paresis or diarrhoea seen in acute cases in south-east Asia. The cyclophosphamide treatment caused a significant decrease in the neutrophil count by 7 d after inoculation in all 15 immunosuppressed pigs, and all were culture positive at necropsy. Eight of the 33 non-treated pigs were culture negative at necropsy. Pigs overcoming the initial phase of infection had more abscess-like nodules that were bacteriologically sterile at necropsy than the pigs with acute cases of melioidosis. *P. pseudomallei* was isolated predominantly from the spleen, lungs and the injection site. Although only one strain was used in this study, it is likely that Australian strains of *P. pseudomallei* are not as virulent as the south-east Asian isolates.

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Introduction

Two forms of porcine melioidosis have been reported. One is the epizootic, mainly acute, form of the disease from which deaths occur mainly in young pigs (Nguyen-Ba-Luong 1956; Thonn et al 1960; Omar et al 1962). The other is the sporadic, mainly chronic, form which is diagnosed only at slaughter (Laws and Hall 1963; Ferry 1973; Little 1979; Thomas 1981). It is the chronic, form of melioidosis that is most common in Queensland (Thomas 1981; Webster et al 1984), although an abortion (Rogers and Anderson 1970) and an acute case of porcine melioidosis (Oids and Lewis 1955) have been recorded. A recent report of melioidosis in intensive pigeries in south-east Queensland (Ketterer et al 1986) where 199 culture positive pigs were condemned at slaughter has emphasised the need to learn more about the pathogenesis of the disease in pigs.

Pigs are relatively resistant to infection with *P. pseudomallei* (Ketterer et al 1986). Production of porcine melioidosis using a single inoculation by oral, nasal, scarring, subcutaneous or intramuscular routes has been ineffective (Stanton and Fletcher 1952; Nguyen-Ba-Luong 1961). The results of 3 oral doses at intervals of 2 d have been equivocal (Nguyen-Ba-Luong 1961; Thomas and Spinks 1983). As the intratracheal route proved successful in preliminary work at this laboratory (Thomas et al unpublished data) using 3x10^4 to 3x10^5 *P. pseudomallei* organisms, this single dose method was adopted for this trial. Weaver pigs were used because of their greater susceptibility to melioidosis than older pigs (Nguyen-Ba-Luong 1961; Omar et al 1962). A number of these trial pigs were immunosuppressed before inoculation.

The main objectives of the study were to assess the susceptibility of pigs to a standard dose of *P. pseudomallei*, and to assess the effect of immunosuppression on the susceptibility.

Materials and Methods

Pigs

Fifty pigs were obtained from a piggy on with no history of melioidosis. The pigs were 7 to 9 weeks old and weighed between 12 and 15kg. Sera from these pigs was negative in the modified cold complement fixation and indirect haemagglutination tests for melioidosis (Thomas and Spinks 1983).

The pigs were housed in an isolation facility. Care was taken to prevent cross-infection.

Inoculum

The inoculums were prepared from a recently isolated, local porcine strain of *P. pseudomallei*, serotype I, grown in tryptic soy broth for 24 h at 37°C. The broth was shaken during incubation to avoid pelicle production. Suspensions of organisms in sterile saline were standardised optically and a final count determined using a Neubauer cytometer. The inoculums were used within 2 h of preparation; 0.5ml of each inoculum was also injected intraperitoneally into guinea pigs to test for virulence.

Experiment

Forty-eight weaner pigs were injected by the intratracheal route with a single dose (1ml) of 5 x 10^4 *P. pseudomallei*, followed by a 1ml rinse of the needles with sterile saline. Two d before inoculation, 500mg doses of cyclophosphamide were prepared as recommended by the manufacturer and injected intravenously into 15 of the trial pigs. Two control pigs were given 1ml doses of sterile saline. The trial lasted 84 d. Two to 6 pigs per week were killed for necropsy. These were chosen at random except when obvious sickness warranted early intervention.

Clinical Observations and Samples

Morning temperatures were recorded daily. Injection sites were carefully examined for lesions, and any exudate was sampled for culture. Blood samples were collected for culture twice weekly for the first 2 weeks after inoculation and weekly thereafter. Total and differential white cell counts were done on blood collected into EDTA. Faeces and urine were collected weekly for culture, but not all pigs were sampled each week. Any nasal discharge was cultured.

Pathology

A complete necropy was performed on each pig. Samples of heart blood, lung, liver, spleen, kidney, brain, cerebrospinal fluid, retropharyngeal lymph nodes, mediastinal lymph nodes, bronchial lymph nodes, mesenteric lymph nodes, tonsils, urine, faeces, injection site (trachea) were collected using aseptic techniques for bacteriological examination. Lesions were selected where present, otherwise pieces of tissue were removed for culture. Lesions present at any other site were also collected for culture.

The number, size and placement of lesions in the tissues were recorded and consistency of the lesions noted.

For histopathology, tissues were fixed in 10% buffered neutral formalin, embedded in paraffin, cut at 3 to 5μm and stained with haematoxylin and eosin.

*Endoxanasta, Astra-Werke A-G Chem. Fabrik Germany*
TABLE 1

Differences observed between 40 experimentally infected pigs (treated or non-treated with cyclophosphamide) that were culture positive for *P. pseudomallei* at necropsy

<table>
<thead>
<tr>
<th>Pigs</th>
<th>Number</th>
<th>Lung*</th>
<th>Splenic</th>
<th>Tissues†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY§</td>
<td>A†</td>
<td>10(100%)</td>
<td>7(70)</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5(20)</td>
<td>4(80)</td>
<td>1.4</td>
</tr>
<tr>
<td>non CY</td>
<td>A</td>
<td>11(73.3)</td>
<td>7(63.6)</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>2(12.5)</td>
<td>13(87.5)</td>
<td>2.2</td>
</tr>
<tr>
<td>CY</td>
<td>A&amp;C</td>
<td>15(93.3)</td>
<td>11(73.3)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1(6.7)</td>
<td>14(66.7)</td>
<td>3.6</td>
</tr>
<tr>
<td>CY &amp; non CY</td>
<td>A&amp;C</td>
<td>21(95)</td>
<td>14(66.7)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>19(81.8)</td>
<td>17(85.0)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* — lung and/or thoracic lymph nodes
† — spleen and/or head lymph nodes
§ — CY = cyclophosphamide
† — A = acute melioidosis
‡ — C = chronic melioidosis
# — (%) = percentage positive

nODULES were raised, cream in colour and well encapsulated. Thirty-nine such nodules were recorded in one spleen. In the wall of the trachea, the lesions developed to form white fibrous capsules (2mm thick) containing cream to green-coloured, semi-gelatinous material. Lesions were rarely seen in the lymph nodes of pigs with acute forms of the disease. The bronchial, mediastinal and retropharyngeal lymph nodes were either normal in size or reddened and enlarged, often to twice their normal size. Single nodules, up to 3cm in size, were occasionally seen in chronic cases.

**Pigs not treated with cyclophosphamide** — visible lesions were seen in 23 of the 33 non-treated pigs at necropsy. Fibrous adhesions were present in the thoracic cavity of one pig and the abdominal cavity of one pig. Eleven pigs had acute melioidosis and 14 pigs had chronic forms of the disease. The gross pathology of the lesions was similar to that seen in the treated pigs. Six of the 11 pigs with acute melioidosis had lesions too numerous to count in the lungs. In one pig, there was complete involvement of all lobes and multiple lesions. Eight non-treated pigs were free of disease at necropsy although 2 of these had sterile lung lesions.

Treatment with cyclophosphamide increased the number of pigs becoming infected (100% treated; 75.8% non-treated), the number of pigs developing acute forms of the disease (66.7% treated; 33.3% non-treated) and the number of pigs with lung and/or thoracic lymph node involvement (73.3% treated; 33.3% non-treated).

The most common sites for nodules in the lungs were the left dorsal diaphragmatic (13), the right dorsal diaphragmatic (11), the left cardiac (5), the right apical (4), the left apical (3) and the right cardiac (3) lobes.

**Histology**

There were two types of lesions seen in the lungs. One was an acute exudative bronchogenic reaction with associated consolidation while the other was a chronic granulomatous productive type lesion represented by nodules. The productive type either followed the exudative lesion or occurred independently. The exudative process showed focal exudation of leucocytes, mainly mononuclear cells and lymphocytes. With age, caseation foci and fibrous tissue appeared and then, a thin capsule of epithelioid cells along with fibrous tissue surrounded the caseated centre. The chronic productive type lesion was formed from initial focal exudation of leucocytes. These leucocytes could not be further differentiated because of intense karyorrhexis and finally complete caseous necrosis. An outer layer of fibrous exudate was frequently present. With age, a thin zone of granulation tissue consisting of macrophages and fibroblasts replaced the fibrous exudate and demarcated the lesions.

TABLE 2
Isolation sites of *P. pseudomallei* in 46 experimental pigs injected by the intratracheal route with 5x10^6 organisms

<table>
<thead>
<tr>
<th>Tissue Description</th>
<th>Pigs treated with cyclophosphamide (15)</th>
<th>Pigs not treated with cyclophosphamide (33)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of culture positive tissues</td>
<td>Number of culture positive tissues with visible lesions</td>
</tr>
<tr>
<td>Inoculation site/trachea</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Lung</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Spleen</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Bronchial lymph node</td>
<td>5</td>
<td>0 (3)*</td>
</tr>
<tr>
<td>Mediastinal lymph node</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Retropharyngeal lymph node</td>
<td>1</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Mandibular lymph node</td>
<td>1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hoof</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tonsils</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Iliac lymph node</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Faeces</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tail</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* — ( ) = number of lymph nodes reddened and enlarged

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**TABLE 3**
Comparison of studies on natural cases of melioidosis in pigs with this experimental study

<table>
<thead>
<tr>
<th>% of tissues from which <em>P. pseudomallei</em> was isolated</th>
<th>Spleen</th>
<th>Lung</th>
<th>Thoracic Disease nodes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.6</td>
<td>13.3</td>
<td>21.3</td>
<td>Chronic</td>
<td>Laws and Hall 1963</td>
</tr>
<tr>
<td>10.0</td>
<td>60.0</td>
<td>40.0</td>
<td>Acute</td>
<td>Omar 1983†</td>
</tr>
<tr>
<td>40.7</td>
<td>40.7</td>
<td>51.9</td>
<td>Chronic</td>
<td>Thomas 1981</td>
</tr>
<tr>
<td>26.4</td>
<td>4.5</td>
<td>0.9</td>
<td>Chronic</td>
<td>Ketterer et al 1986</td>
</tr>
<tr>
<td>34.0</td>
<td>7.0</td>
<td>40.0</td>
<td>Chronic</td>
<td>1990</td>
</tr>
<tr>
<td>60.0</td>
<td>90.0</td>
<td>70.0</td>
<td>Acute (CY)†</td>
<td>This study 1990</td>
</tr>
<tr>
<td>60.0</td>
<td>20.0</td>
<td>0.0</td>
<td>Chronic (CY)</td>
<td></td>
</tr>
<tr>
<td>63.6</td>
<td>81.8</td>
<td>45.8</td>
<td>Acute (non-CY)†</td>
<td></td>
</tr>
<tr>
<td>71.4</td>
<td>14.3</td>
<td>0.0</td>
<td>Chronic (non-CY)</td>
<td></td>
</tr>
</tbody>
</table>

* — CY = cyclophosphamide treated pigs
† — non-CY = non-cyclophosphamide treated pigs
‡ — south-east Asian study

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The productive type nodules of the spleen and liver had well developed capsules. The thickness of this capsule was related to the age of the nodules.

**Haematology**

There was a marked neutrophilia in all infected pigs 3 d after inoculation. In the treated pigs, the neutrophil count returned to ‘normal’ levels by 7 d after inoculation, while in the non-treated pigs, the neutrophil count remained high until 21 d after inoculation. There was a slight drop in the lymphocyte count in both sets of pigs from 7 to 14 d after inoculation, but still within the ‘normal’ range.

**Bacteriology**

Table 1 shows the percentage of the 40 culture positive pigs that had *P. pseudomallei* isolated from the lung and/or associated lymph nodes and from the spleen and/or lymph nodes of the head. The average number of infected tissues per pig is also indicated.

Table 2 shows the isolation sites for *P. pseudomallei*, including the number of culture positive tissues showing visible lesions. Results for both treated and non-treated pigs are shown.

**Pigs treated with cyclophosphamide** — all 15 treated pigs were culture positive at necropsy. The average number of culture positive tissues was 2.9 (range 1 to 6). Five faecal and 18 urine samples collected during the trial were culture negative. One pig had a culture positive blood sample 21 d after inoculation, while another pig had a sterile lesion in the lung when necropsied 77 d after inoculation.

**Pigs not treated with cyclophosphamide** — *P. pseudomallei* was isolated from 25 of the 33 non-treated pigs at necropsy. The average number of culture positive tissues was 2.2 (range 1 to 7). Twenty-six faecal and 59 urine samples collected during the trial were culture negative. Two of the 8 culture negative pigs had sterile lesions in the lungs, and 2 culture positive pigs had sterile lesions in either the spleen or throat. All sterile lesions were in pigs necropsied 70 to 84 d after inoculation. There were 5 culture positive blood samples. These were from 3 pigs at 14 d after inoculation, one pig at 28 d after inoculation and one pig at 35 d after inoculation.

**Discussion**

This study confirmed preliminary work at this laboratory that ≥5X10^5 *P. pseudomallei* organisms injected by the intratracheal route would produce melioidosis in pigs. Forty of the 48 experimentally inoculated pigs developed the disease.

Natural infection by *P. pseudomallei* occurs through ingestion, inhalation or wound entry of soil or soil-contaminated food and water, *P. pseudomallei* being a soil saprophyte (Thomas et al 1979). Ingestion and wound entry generally lead to spleen and head lymph node involvement (Laws and Hall 1963; Thomas et al 1981) while aerosol inhalation (from high pressure sprayers and pressure nipple drinkers) leads to lung and thoracic lymph node involvement (Ketterer et al 1986). The chronic form of the disease is more common in Queensland (Laws and Hall 1963; Thomas 1981).

Twenty-one acute and 19 chronic cases of melioidosis developed during our trial. The larger numbers of acute cases seen was due to 2 major factors; the method of injection and the use of cyclophosphamide as an immunosuppressant.

Our intratracheal inoculation provided both aerosol (forbice injection by needle) and ingestion (initial swallow plus possible reinfection from tracheal lesions) routes of infection. Where aerosol infection played the major part, acute cases of bronchopneumonia ensued, with associated involvement of the thoracic lymph nodes. Where ingestion was the major route of entry, chronic cases were more common and the spleen was more often affected than the lung (Table 1).

Treatment with cyclophosphamide did not significantly affect the clinical signs, histopathology and bacteriology as seen in the non-treated pigs. However, it did increase the number of pigs affected and the number of pigs with acute forms of the disease. This is possibly due to the marked drop in the neutrophil count at 7 d after inoculation in the treated pigs. Cyclophosphamide, a cytotoxic drug, is known to act against
cells of the immune system, particularly polymorphonuclear cells and macrophages (Buhles and Shifrine 1979; Tatsukawa et al 1979; Muneer et al 1988) which are important in the primary stages of infection (Sawada et al 1987).

The signs of acute melioidosis seen in pigs in south-east Asia, such as nasal and ocular discharge, paresis of the hind legs and diarrhoea (Nguyen-BA-Luong 1956; Thonn et al 1960; Omar et al 1962) were not seen in this trial. Table 3 compares results of spleen, lung and thoracic lymph node involvement in pigs with melioidosis in field and experimental cases in Queensland and south-east Asia. It would appear that strains of P. pseudomallei isolated in Queensland may not be as virulent as the Asian isolates.

The histopathology seen in our trial was similar to that described by Omar (1963) in field cases of porcine melioidosis. However, he found that the apical and cardiac lobes were the most commonly affected lung lobes in the natural cases he investigated. In contrast, the diaphragmatic lobes were the most commonly affected lobes in 13 of the 14 pigs with lung lesions in our experimental work. The forcible injection of P. pseudomallei into the trachea of pigs held vertically, head upwards, allowed direct entry into the diaphragmatic lobes. No changes in the thoracic lymph nodes, other than oedema and congestion, were evident in acute cases and this is in agreement with the observation on acute melioidosis made by Omar (1963).

The age of the lesions could be deduced from the size of the nodules, the degree of encapsulation and the consistency of the contents. Very few nodules were seen in pigs killed at 3 to 7 d after inoculation. At 7 to 14 d after inoculation, the roughly spherical nodules averaged 0.4cm in diameter. After 14 d, nodules were found to vary from 0.5 to 3.0cm. In the lungs, nodules were either large, discrete and well-encapsulated with semi-solid, tenacious contents, or small and tending to coalesce into areas up to 8.5cm which were filled with caseated material. The older lesions in the spleen were multiple, discrete, large well-encapsulated lesions that protruded above the surface of the organ. As the lesions aged, the contents turned from moist and caseous to dry and crumbling. Older lesions were often sterile. Five pigs had sterile lesions suspected of being melioidosis in regression. Sterile lesions are not uncommon in natural infections in older pigs (Omar et al 1962) and goats (Olds and Lewis 1954; Thomas et al 1988). In this trial, the sterile lesions were found in pigs killed between 70 and 84 d after inoculation.

P. pseudomallei was isolated from the faeces of only one pig, emphasising the fact that transmission of melioidosis by this means is rare (Nouvel et al 1976; Thomas et al 1988), even though faecal spread of P. pseudomallei in horses in France has been documented (Gailimand and Dodin 1982).

Two pigs had culture positive lesions in unexpected sites. One pig had a nodule in the right hoof (other sites being lung, spleen and retropharyngeal lymph node) and another pig had a nodule near the tail (other sites being the injection site and the spleen). A thorough necropsy is recommended when melioidosis in pigs is suspected.

This trial has supplied relevant data towards the understanding of the pathogenesis of P. pseudomallei in pigs. However, it was not a true indication of what occurs in the field where the chronic form of melioidosis is more common. Further work, involving use of a lower dose of organisms more consistent with field concentrations (up to 5x10^5/g — Thomas and Forbes-Faulkner 1981) and use of an aerosol or multiple dose oral inoculation technique to avoid reinfection from tracheal lesions, could be worthwhile.

The spleen will be the major organ involved in field cases where oral and wound entry are normal methods of ingress for P. pseudomallei. A high incidence of lung and thoracic (especially bronchial) lymph node involvement would indicate an aerosol infection. Any stress on the pigs, especially weaners, will increase the risk of infection.

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