PATHOLOGICAL AND BIOCHEMICAL FEATURES OF LEGIONELLA PNEUMOPHILA INFECTION IN GUINEA-PIGS

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SUMMARY. The main pathological feature of experimental legionellosis produced by the intraperitoneal inoculation of guinea-pigs was a fibrinopurulent peritonitis, especially over the liver and spleen. Foci of necrosis were present in these organs from the second to seventh day after infection. Early biochemical changes in the serum included significant decreases in the concentration of zinc and iron, and increases in copper and triglycerides. Phenylalanine to tyrosine ratios increased strikingly, but free amino acid decreased slightly. The total protein concentration did not change, but acute-phase proteins increased. Serum lysozyme activity increased as leucocytosis developed but fell during the subsequent leucopenia. In the later stages of the disease the activity of alkaline phosphatase, γ-glutamyl transpeptidase, and creatine kinase decreased; that of dehydrogenases and transaminase increased.

INTRODUCTION

Since the first description of Legionnaires' disease (LD) in the USA (Fraser et al., 1977) cases have been recognised with increasing frequency in many countries, including Britain, and the disease is clearly much more widespread than was previously supposed. LD was originally considered to be exclusively a pulmonary disease, but it is now evident that in a high proportion of patients the causative bacterium, Legionella pneumophila, may spread to other organs such as the liver, spleen and kidneys. This results in a systemic illness in which patients frequently show biochemical abnormalities of the serum associated with hepatic and renal failure (Alexander and Dismukes, 1979; Cordes and Fraser, 1980; Saleh et al., 1980). The published data have, in the main, been derived from acutely ill patients who have received treatment; biochemical information on the early stages of the disease in untreated patients is limited. Although Chandler et al. (1979b) described pathological changes in guinea-pigs with experimental legionella infection they did not investigate the biochemical response to infection.

Interrelated metabolic changes associated with tissue damage occur in man and other animals as the first recognisable response to acute pyrexial infections (Powanda et al., 1975; Hambleton et al., 1977). These changes may be initiated by circulating
substances elaborated by leucocytes in response to inflammatory processes (Wannemacher et al., 1972a); they include early changes in the concentrations of serum trace metals (Wannemacher et al., 1972a; Powanda et al., 1975; Beisel, 1976; Hambleton et al., 1977, 1978) and amino acids that may be concerned with the subsequent synthesis of acute-phase proteins (Wannemacher et al., 1972b; Powanda et al., 1975). Increases in the serum phenylalanine to tyrosine ratio also often precede inflammatory changes (Wannemacher et al., 1976; Hambleton et al., 1978). Changes in serum enzyme concentrations also characterise many microbial infections. For example, decreased alkaline phosphatase activity is an early feature of tularemia in animals (Hambleton et al., 1977, 1978); and increase in dehydrogenase, transaminase, and creatine kinase occur during typhoid fever (Wannemacher et al., 1972a), leptospirosis (Arean, 1962), and tularemia (Hambleton et al., 1977, 1978). The monitoring of such biochemical changes can be of diagnostic and prognostic value (Hambleton et al., 1978) and may also provide guidelines for supportive biochemical therapy.

The present work was designed to study the systemic effects and biochemical changes occurring in guinea-pigs infected with *L. pneumophila*.

**Materials and methods**

*Bacterial strain.* The Corby strain of *L. pneumophila* (serogroup 1) was kindly provided by Dr R. A. Swann, Dept of Virology, John Radcliffe Hospital, Headington, Oxford, in the form of infected guinea-pig spleen.

Spleens from guinea-pigs inoculated 3 days earlier were placed in sterile polyethylene bags with approximately 4 volumes of nutrient broth and homogenised for 1 min in a Colworth stomacher 400 (Model BA 6021, A. J. Seward and Co Ltd, 6, Stamford St, London, SE1 9UG). Tissue debris was removed by centrifugation (250 × g, 5 min) and the supernatant fluid used for inocula.

Because the strain of *L. pneumophila* in tissue homogenate did not grow on charcoal-yeast extract agar (Feeley et al., 1979) or enriched blood-agar (Greaves, 1980), the presence of the organism was confirmed by indirect fluorescent-antibody staining with antiserum to the organism produced in rabbits and a goat anti-rabbit serum labelled with fluorescein isothiocyanate.

*Animals.* Forty-five female Dunkin-Hartley guinea-pigs of category-4 status weighing 250-300 g were infected by the intraperitoneal injection of 2.5 ml of inoculum prepared as described above. Another 10 animals served as uninfected controls.

*Blood samples.* Guinea-pigs were divided into groups of 5-10. Blood (5-6 ml) was removed by cardiac puncture from each animal in a group, a different group being sampled on each day. Blood samples from control and infected animals were processed as described by Hambleton et al. (1979) and the separated filtered sera were stored at −20°C. Each infected animal was sampled only once, but a second blood sample was taken from control (uninfected) animals 4 or 5 days after the first cardiac puncture.

*Histopathology.* Infected and control animals were killed with ether on days 1, 2, 3, 4 and 7, and necropsies were done immediately. Portions of the following tissues were removed and fixed for histopathology in 10% (v/v) neutral buffered formalin: brain, heart, lung, liver, spleen, kidney, small and large intestine, mesenteric lymph nodes, urinary bladder, mesentery, ovaries, and uterus. The tissues were processed by standard methods and embedded in paraffin wax. Sections 5 μm thick were stained by haematoxylin and eosin and selected sections were also stained by the following methods: Gram’s, as modified by Bartholomew (1962) and by Brown and Brenn (1931); gram-methyl pyronin-light green (GMP-LG) (Sowter and McGee, 1976); Warthin and Starry’s (1920) silver impregnation; and Giemsa’s.

Portions of liver, spleen and lung were also taken and homogenised for the detection of *L. pneumophila* by immunofluorescence microscopy.

*Serum analyses.* Concentrations of calcium, copper, iron, magnesium, potassium, sodium
and zinc were determined with either flame or flameless atomic-absorption spectrophotometry as described by Hambleton et al. (1977, 1980). The activities of alkaline phosphatase (AP), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), lactate dehydrogenase (LDH), $\alpha$-hydroxybutyrate dehydrogenase ($\alpha$-HBDH), creatine kinase (CK), and $\gamma$-glutamyl transpeptidase, and the concentrations of glucose, triglycerides, total protein, urea, and uric acid were measured with test kits (Boehringer Corp. Ltd, Bell Lane, Lewes, Sussex); and sorbitol dehydrogenase (SDH) activity was measured by the method of Rose and Henderson (1975) in a Clinicon 2086 Reaction Rate Analyser with a Clinicon 2082 Kinetic Data Processor (BCL Clinicon, Bell Lane, Lewes, Sussex). Serum lysozyme activity was estimated by the method of Osserman and Lawlor (1966). The concentration of free amino acids was determined as described by Hambleton et al. (1977). Serum proteins were separated by cellulose acetate electrophoresis (apparatus from Gelman Hawksley Ltd, Lancing, Sussex) and the concentration of orosomucoid ($\alpha$-1 acid glycoprotein) was measured by the method of Neuhaus, Balegno and Chandler (1966).

**Haematology.** Total red- and white-cell counts, mean corpuscular volume, and haematocrit were measured by methods described by Hambleton et al. (1977).

**RESULTS**

**Clinical signs**

One day after infection most animals had increased temperatures (40.3–40.8°C, ruffled fur, and a serous ocular discharge, and were dull and anorexic. By day 2 some were emaciated. On the third and fourth days several died, and others were moribund with a subnormal temperature. Those that survived this acute phase of the disease began to eat and gradually improved in condition.

**Necropsy findings**

The only significant lesion was fibrinopurulent peritonitis, principally affecting the surface of the liver and spleen. In some animals the exudate was also evident on the serosal surfaces of the intestines and uterus. Cloudy and bloodstained peritoneal fluid was often present in quantities of up to 5 ml. Lesions were not found in the organs of control animals.

**Histopathological findings**

In the liver and spleen on days 2–7 there were numerous foci of parenchymal necrosis often associated with a layer of fibrinopurulent exudate on the capsule. Some of the foci contained neutrophils and histiocytes undergoing lysis and the spleen was often diffusely infiltrated by neutrophils. Many of the liver lesions had an outer area of early coagulative necrosis.

The inflammatory exudate on the serosal surfaces of the liver, spleen, mesentery, urinary bladder, intestines and uterus was made up of fibrin, neutrophils, macrophages, and cell debris. A striking feature was extensive lysis of the cells in the exudate in many areas. The serosa and muscular layers of the intestines and uterus were occasionally infiltrated by inflammatory cells which had penetrated from the surface. In animals with extensive peritonitis, the mesenteric lymph nodes also contained masses of lysed neutrophils.

Legionellae could not be demonstrated in lesions or exudate by the tissue gram
stains used (Bartholomew, Brown-Brenn, GMPLG); bacteria were demonstrated by Warthin and Starry's silver impregnation method and Giemsa's method but these do not selectively stain for Legionella. Examination of smears from infected guinea-pigs stained by indirect immunofluorescence showed L. pneumophila in blood, liver and spleen, free and within cells. Peritoneal exudate contained many within macrophages but fewer within polymorphonuclear leucocytes.

**Changes in blood components**

The values reported were determined by averaging those obtained from individual guinea-pigs sampled before, and at intervals after, infection. Significant changes in several blood components were observed in infected animals but not in uninfected controls.

**Haematology**

Striking changes in numbers of red blood cells, mean corpuscular volume or haematocrit (normal values $4.13 \pm 0.1 \times 10^6$/mm$^3$, $48 \pm 1.2$ µm$^3$, and $23.8 \pm 0.6$% respectively) were not observed in infected animals. However, some animals developed a leucocytosis by the first day after infection ($12-19 \times 10^3$ leucocytes/mm$^3$), but thereafter a leucopenia developed and reached its maximum by day 3. These changes were less apparent in other animals and so the effects appear less pronounced in the data shown in table I. In some instances animals became severely ill but nevertheless recovered and were found to have a severe leucocytosis ($16-3 \pm 0.99 \times 10^3$ leucocytes/mm$^3$) 7 days after infection.

**Serum metals**

Among the earliest changes to occur were those affecting the trace metals. Thus, serum copper concentrations were elevated (by 50% of their pre-infection value; see table I) on the first day after infection, and continued to increase thereafter (350% of their pre-infection value by day 3). In contrast, the iron and zinc concentrations decreased on day 1 to about 75% of their original value, and continued to fall to about 50% by day 3 (table I).

Calcium concentrations were depressed to about 80% of their original value throughout the first 4 days, but although magnesium, sodium, and potassium decreased slightly as the disease progressed, the values were never more than 10–15% below the pre-infection values, and may not represent significant decreases (table I).

In animals still surviving 7 days after infection, the serum copper concentrations were still elevated (250% of the pre-infection value), and iron and zinc were slightly depressed (60% and 85% of the pre-infection values, respectively); the values for other metals were normal.

**Serum proteins and free amino acids**

Protein concentrations did not change much during the course of the disease (table II). However, cellulose acetate electrophoresis showed that, 2 days after infection, the
### Table I

**Changes in some whole blood and serum components of guinea-pigs infected with Legionella pneumophila**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mean value (± SE) for groups of 5–10 animals sampled at the stated intervals (days) after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>White blood cells (× 10³/mm³)</td>
<td>6.5±0.39</td>
</tr>
<tr>
<td>Copper (mg/L)</td>
<td>0.41±0.02</td>
</tr>
<tr>
<td>Iron (mg/L)</td>
<td>1.86±0.17</td>
</tr>
<tr>
<td>Zinc (mg/L)</td>
<td>1.65±0.12</td>
</tr>
<tr>
<td>Calcium (mMol/L)</td>
<td>2.41±0.14</td>
</tr>
<tr>
<td>Magnesium (mMol/L)</td>
<td>1.04±0.14</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>135.0±2.30</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>5.72±0.30</td>
</tr>
<tr>
<td>Triglycerides (g/L)</td>
<td>1.18±0.23</td>
</tr>
</tbody>
</table>

**Serum**

### Table II

**Serum protein and free amino-acid concentrations, and body temperatures of guinea-pigs infected with Legionella pneumophila**

<table>
<thead>
<tr>
<th>Serum component</th>
<th>Mean value (± SE) for groups of 5–10 animals sampled at the stated intervals (days) after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (g/L)</td>
<td>47.3±0.9</td>
</tr>
<tr>
<td>Orosomucoid (α-1 acid glycoprotein) (mg/ml)*</td>
<td>0.86</td>
</tr>
<tr>
<td>Free amino acids (mMol/L)</td>
<td>3.56±0.17</td>
</tr>
<tr>
<td>Phenylalanine to tyrosine ratio</td>
<td>1.20±0.11</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>39.0±0.20</td>
</tr>
</tbody>
</table>

* Analyses performed on pooled sera.

α globulins had increased in some animals; and by days 3 and 4 striking increases in α and β globulins were apparent in all infected guinea-pigs. In some animals a slight decrease in albumen occurred late in the disease. Orosomucoid (α-1 acid glycoprotein) also increased as the disease progressed (table II). Concentrations of acute-phase globulins remained high in animals still surviving 7 days after infection.

By day 4 the concentrations of the free amino acids were about 15% below the pre-infection values (table II); and by day 7 about 30% below in the animals still alive. In contrast, the ratio of phenylalanine to tyrosine increased dramatically during the first 24 h of infection; it continued to rise, and remained elevated, in lethally infected animals (table II). In animals still alive on day 7 the ratio had fallen to a normal value (0.98±0.1).

### Serum enzymes

The activity of several enzymes changed but, except for lysozyme, not before day 2 (table III). Thus SDH activity was elevated on days 2 and 3, and even higher on day 4.
ALAT activity was strikingly elevated, and ASAT activity rather less so on day 4; neither enzyme showed any change before the fourth day. In contrast the activities of AP and γ-GT fell 3–4 days after infection. CK activity was elevated in some, but not all, animals on day 2 but was low on day 4. LDH and α-HBDH activity appeared to be slightly elevated on day 4. Although the activity of LAP fluctuated, obvious trends were not apparent.

In contrast to the activity of other serum enzymes, lysozyme activity was elevated on the first day after infection but thereafter decreased, approaching normal levels by days 3 and 4 (table III).

The enzyme activity in animals still surviving at 7 days did not differ significantly from that found before infection.

**Table III**

*Serum enzyme activities in guinea-pigs infected with Legionella pneumophila*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mean value (±SE) for groups of 5–10 animals sampled at the stated intervals (days) after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>310.9 ± 40.1</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>93.2 ± 25.0</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>129.1 ± 27.0</td>
</tr>
<tr>
<td>Creatine kinase (U/L)</td>
<td>425.3 ± 76.4</td>
</tr>
<tr>
<td>γ-Glutamyl transpeptidase (U/L)</td>
<td>20.1 ± 2.9</td>
</tr>
<tr>
<td>α-Hydroxybutyrate dehydrogenase (U/L)</td>
<td>627.9 ± 82.8</td>
</tr>
<tr>
<td>Creatine dehydrogenase (U/L)</td>
<td>2798 ± 1052</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>598.8 ± 32.5</td>
</tr>
<tr>
<td>Leucine aminopeptidase (U/L)</td>
<td>1.71 ± 0.54</td>
</tr>
<tr>
<td>Lysozyme (g/L)</td>
<td>121.9 ± 21.4</td>
</tr>
</tbody>
</table>

**Other serum components**

Triglyceride concentrations, which were elevated by day 1, continued to increase and remained high in infected animals (table I). Changes were not detected in urea, uric acid, or glucose (normal values 203 ± 2, 13.8 ± 2.4, 101 ± 8 mg/L respectively).

**DISCUSSION**

The histopathological changes observed closely resemble those described previously by Chandler et al. (1979b) for legionella-infected guinea-pigs; these authors did not study biochemical features of the disease. A mild to moderate leucocytosis occurs in human cases of Legionnaires' disease (Fraser et al., 1977; Lattimer and Rhodes, 1978; Cordes et al., 1980; Saleh et al., 1980), and Pontiac fever (Cordes and Fraser, 1980), and phagocytosis of legionellae by macrophages in human tissues has been observed (Glavin, Winn and Craighead, 1979; Chandler et al., 1979a). Increased
serum lysozyme activity may occur either during phagocytosis or as a consequence of the degradation of phagocytes (Berendt, Long and Abeles, 1977; Hambleton et al., 1978). Thus the early increase in lysozyme observed in the present study may reflect the phagocytosis that occurs during the period of leucocytosis. The leucopenia that developed in the later stages of the disease was probably due to massive exudation into the peritoneum. It was not accompanied by high serum lysozyme concentrations, suggesting that phagocytic degradation was minimal at this time. Although leucopenia has been observed in some human legionella infections, it is uncommon (Fraser et al., 1977).

Little information is available on serum metal concentrations in human legionellosis, although hyponatraemia has been reported in some patients (Cordes and Fraser, 1980). In the present study in guinea-pigs, calcium clearly decreased and sodium, potassium and magnesium tended to fall slightly as the disease progressed. This might seem to indicate mild renal dysfunction, but there was no histopathological evidence and the serum urea concentrations remained unchanged. In contrast, renal complications have sometimes been reported in human legionellosis (Harvey, Quirke and Warren, 1980; Saleh et al., 1980; Williams et al., 1980), although the exact nature and cause of the renal lesion are not yet known.

In guinea-pigs striking changes occurred in serum trace metals, amino acids and proteins, resembling those induced by other acute bacterial and viral infections of man and animals (Wannemacher et al., 1972a; Powanda et al., 1975; Beisel, 1976; Hambleton et al., 1977, 1978). The decreases in zinc may have been associated with the decrease in free amino acids observed in the later stages of the disease; a similar association is often observed in experimental infections of man and other animals (Wannemacher et al., 1972a, 1975). This may be related to the synthesis of acute-phase proteins (Powanda et al., 1975; Berendt et al., 1977; Powanda, 1977), although the latter may not necessarily result in a measurable decrease in amino acids (Hambleton et al., 1977, 1978). Synthesis of acute-phase protein was an obvious feature in the present study; increases occurred in \( \alpha \) and \( \beta \) globulins, including orosomucoid and ceruloplasmin, synthesis of the latter being reflected in increased copper concentrations.

An increase in the ratio of serum phenylalanine to tyrosine, together with a decrease in serum amino acid, is a good indicator of the onset of an inflammatory state (Wannemacher et al., 1976), although it does not always occur (Hambleton et al., 1977). In this study it occurred as soon as pyrexia became severe.

Hepatic abnormalities in human legionellosis are indicated by increases in serum transaminase and other liver enzymes (Fraser et al., 1977; Lattimer and Rhodes, 1978; Alexander and Dismukes, 1979; Cordes et al., 1980). The activity of serum transaminase and SDH increased in legionella-infected guinea-pigs, probably as a result of the observed hepatic necrosis. AP activity may be mildly elevated in human patients (Fraser et al., 1977; Lattimer and Rhodes, 1978; Saleh et al., 1980), but in this study it decreased as the disease progressed. In the light of earlier studies (Hambleton et al., 1977, 1978) this decrease may further reflect hepatic damage.

The slight increase in LDH and \( \alpha \)-HBDH activity seen shortly before death may indicate the late onset of more widespread tissue necrosis. Varying degrees of increased LDH activity occur in human legionellosis (Lattimer and Rhodes, 1978; Saleh et al., 1980). CK activity may increase in some human infections (Lattimer and
Rhodes, 1978; Harvey et al., 1980; Saleh et al., 1980); transient increases occurred in a few guinea-pigs. The observed decreases in the activity of CK and γ-GT late in the disease were unexpected because the appearance of these enzymes in serum is caused by tissue breakdown.

The increased serum triglyceride concentrations were similar to those observed in other experimental infections (Hambleton et al., 1977, 1978); they probably resulted from an enhanced rate of synthesis in the liver (Canonico et al., 1977), though in this study fatty changes in the liver were absent.

Bacteriaemia was not detected in this study although other strains of L. pneumophila may give rise to a bacteriaemia in guinea-pigs 1 or 2 days after infection (R. B. Fitzgeorge, unpublished). Legionellosis in man is not limited to the respiratory tract but commonly has a bacteriaemic phase and causes systemic disturbance (Fraser et al., 1977; Alexander and Dismukes, 1979; Cordes and Fraser, 1980; Williams et al., 1980). Thus, although the experimental infection described in this report does not affect the respiratory tract, many of its features reflect aspects of the human disease. Unsuccessful attempts were made in this study to induce respiratory infection in guinea-pigs by intranasal instillation with the Corby strain of L. pneumophila.

The precise pathogenic mechanisms in legionellosis are not known. In the present study the presence of L. pneumophila was strongly associated with disease-affected tissues and peritoneal exudate, and the observed lesions might have resulted, at least in part, from localised bacterial multiplication. L. pneumophila produces an endotoxin-like material (Wong et al., 1979; Fumarola, Monno and Monno, 1980), a haemolysin (Baine et al., 1979), a protease (Muller, 1980), and a cytotoxin (Friedman, Iglewski and Miller, 1980). The pathogenic significance of toxic factors is not known, but it is possible that they contribute to virulence and to the causation of lesions, in the locality of the infecting organisms and at more distant sites.

REFERENCES

BARTHOLOMEW, J. W. 1962. Variables influencing results, and the precise definition of steps in gram staining as a means of standardizing the results obtained. Stain Technology, 37, 139–155.


